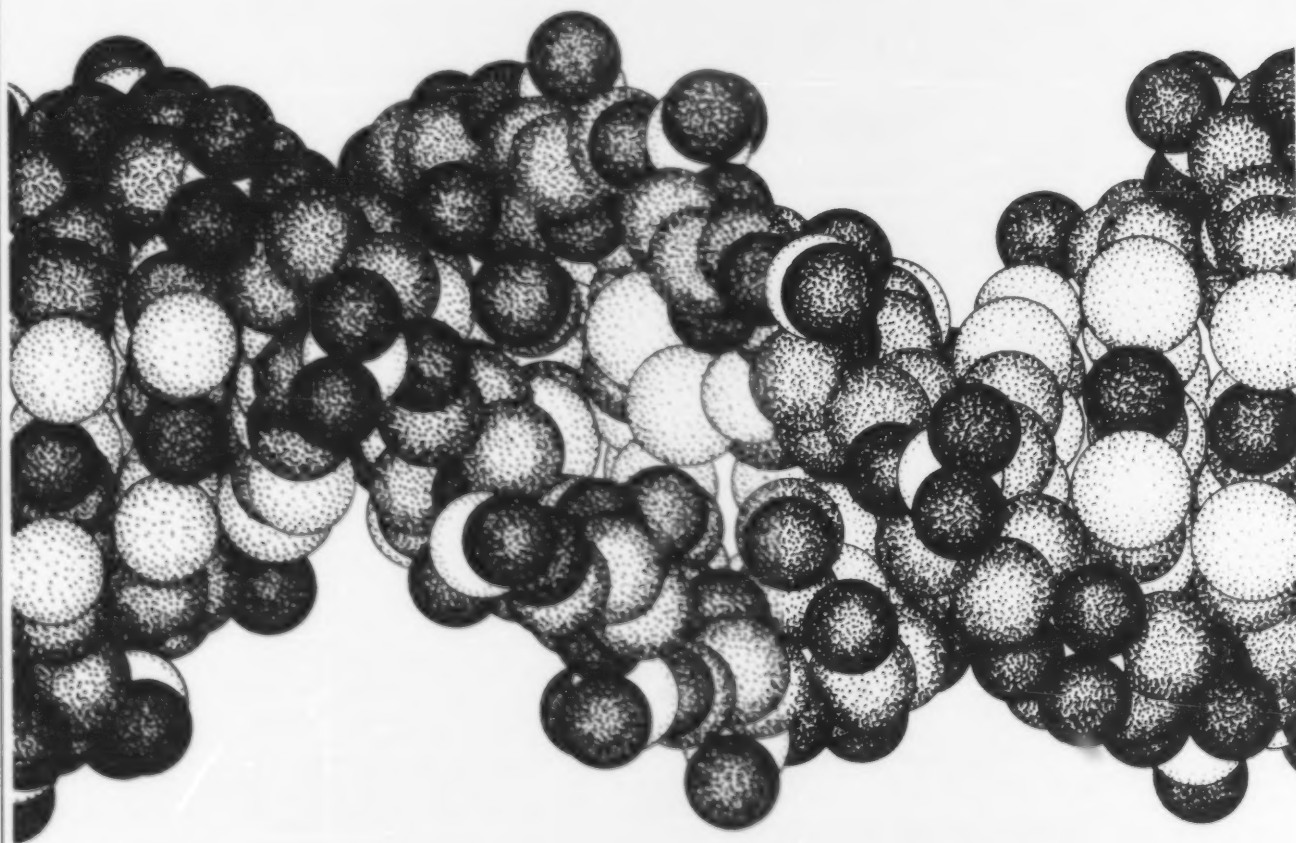




Basics of plant genetic engineering and its potential applications to tree species

P.J. Charest and M.-F. Michel

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BASICS OF PLANT GENETIC ENGINEERING AND ITS POTENTIAL APPLICATIONS TO TREE SPECIES

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aux espèces d'arbres.**

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This work is dedicated to the memory of the late Marie-France Michel

ABSTRACT

The genetic engineering of plants has advanced phenomenally in the eight years since the first regeneration of a transgenic plant. The present information report reviews the state-of-the-art in the techniques of genetic engineering for plants, covering both direct and indirect gene transfer methods. A comprehensive list of all plant species in which transgenic plants have been recovered has been compiled and criteria for proof of stable integration are given. As well, agronomic characters used for genetic engineering are reviewed. The application of technologies developed with crop plants to tree species is discussed, starting with a survey of genetic transformation results followed by a discussion on problems associated with tree species. The use of agronomic characters for genetic engineering of trees is covered, and the report concludes with sections on the ecological impacts of genetically engineered trees, regulations for testing and release of transgenic trees, and a brief overview of research activities in tree genetic transformation in Canada.

RÉSUMÉ

Le génie génétique des plantes avance à pas de géant depuis huit ans. Le début de cette période est marqué de la première régénération d'une plante transgénique. Le présent rapport d'information passe en revue les procédés les plus modernes de génie génétique des plantes. Tous les deux aspects, les méthodes directes et indirectes y sont inclus. Une liste détaillée énumère toutes les espèces végétales chez lesquelles les plantes transgéniques ont été recouvrées. Les critères pour les épreuves d'intégration stables y figurent également. En outre, on examine des caractéristiques agronomiques qui sont utilisées pour le génie génétique. L'analyse comprend l'application de telles technologies génétiques aux arbres qui étaient élaborées antérieurement pour les produits agricoles. On étudie les résultats de la transformation génétique, ensuite, on discute des problèmes qui sont reliés aux espèces d'arbres. L'emploi des caractéristiques agronomiques au génie génétique des arbres est aussi décrit. La dernière section traite les impacts écologiques que peuvent avoir des arbres assujettis au génie génétique ainsi que les règlements qui s'appliquent en cas de tests et de commercialisation des arbres transgéniques. Le rapport s'achève par un aperçu des activités de recherche dans le domaine de la transformation génétique des arbres au Canada.

1. INTRODUCTION

Plants can regenerate in culture from single isolated cells into complete organisms identical to the parent. This unique experimental capacity makes them very attractive for molecular biology research. Plant cells can be genetically transformed with exogenous DNA and the regenerated transgenic plants, with the foreign DNA integrated into their genomes, can be used for studying gene expression and gene regulation.

Production of transgenic plants is relatively new (section 3.4.1) and most of them were obtained with model plant systems such as *Nicotiana tabacum* (tobacco) and *Petunia hybrida* (petunia), which are both members of the Solanaceae family. Transgenic plants from crop and tree species are more difficult to produce due to inefficiencies in culture systems (section 3.4.1). The most advanced technology for the genetic transformation of plants employs the phytopathogenic bacterium *Agrobacterium tumefaciens* (section 3.3.1) (which induces crown gall disease). The molecular basis of this disease is the transfer of plasmid DNA (T-DNA or transfer DNA) to the plant cell genome and its subsequent expression, which induces abnormal proliferation of the transformed cell (section 3.3.1). Application of this technology to plants is limited by the host range of the bacteria, but alternative methods of transformation have been developed that are complementary to the *Agrobacterium* transformation (section 3.2).

The genetic transformation of plants has been achieved with selectable marker genes, such as antibiotic resistance genes (section 3.4.2) and, recently, with genes coding for agronomic traits. Characters such as resistance to viral diseases (section 4.1), insect pests (section 4.2), heavy metal contamination (4.3), herbicides (4.4), and fungal infection (4.5) have been transferred to model plant systems, such as *N. tabacum*, *P. hybrida*, *Lycopersicon esculentum* (tomato), and *Arabidopsis thaliana*.

The application of genetic engineering to tree species (coniferous and deciduous) offers new opportunities for gene regulation, gene expression, and genetic improvement. However, little work has been done with tree species (see section 5). Genetically transformed tree species

have only been obtained with poplar (*Populus* spp.), walnut (*Juglans regia*), apple (*Malus pumila*), and neem (*Azadirachta indica*) (section 5.1). Although techniques needed to advance this field of research have been developed with other systems (mammals, bacteria, yeasts, and crop plants), much work is needed to apply these techniques to tree species.

The present information report should help forestry researchers get a feel for the state-of-the-art in the field of plant genetic engineering, as well as appreciate the potential of this research with respect to tree species.

2. MAJOR CONCEPTS AND TERMINOLOGY

2.1 Terminology

A characteristic of a rapidly evolving area of research is often confusion about terminology. To ameliorate this, general terms will be defined in this section. The first expression with the broadest meaning is *genetic manipulation*, which includes any manipulation or modification that can be performed on the genome of an organism. Genetic manipulation covers plant breeding, somatic hybridization, and genetic transformation. *Genetic transformation* is more restricted and refers to the transfer and integration of exogenous DNA to the genome of an organism. This is done with or without gene isolation or modification and includes chromosome transformation, *Agrobacterium*-induced tumorigenesis, and *genetic engineering*. The latter expression will be used in this book to refer to the introduction of an engineered (or modified) piece of DNA into the genome of a living organism to deliberately produce a specific characteristic. The organism may or may not be regenerated in its original form.

2.2 Milestones in plant molecular biology and plant genetic engineering

The first evidence for this type of genetic manipulation was published by Wullems et al. (1981). In their study, *N. tabacum* teratomas (differentiated tumorous tissues produced by *A. tumefaciens* transformation) were grafted onto the stem of normal *N. tabacum* plants to obtain fertile flowers and seeds. The transferred genes were not modified and the plants regenerated were essentially chimeras. The transferred DNA was

transmitted to the progeny. This work was preceded by the regeneration of transgenic *N. tabacum* obtained from *Agrobacterium rhizogenes*-transformed hairy roots (Ackermann 1977), but Ackermann's work went almost unnoticed because the nature of *A. rhizogenes* transformation was not understood at the time. It was only in 1983 that the first critical evidence of plant genetic engineering was published (Barton et al. 1983). A modified gene located on the T-DNA of the *A. tumefaciens* tumour-inducing (Ti) plasmid was introduced into *N. tabacum*. Normal transgenic plants (morphologically) were regenerated and the introduced DNA was transmitted to the progeny in a Mendelian fashion.

Early scientific milestones opened the way for these breakthroughs in technology. Table 1 lists what the authors believe to be some of the most important steps in the evolution of plant molecular biology and plant genetic engineering. Basic research on *Agrobacterium* generated the knowledge required for recovery of transgenic plants using this natural gene transfer mechanism. Other basic tools for modifying crop plants have evolved and a fair understanding of plant gene structure has been acquired. However, little is

known about plant biochemistry, its relationship with gene expression, and how these principles determine plant quality and productivity.

3. GENETIC TRANSFORMATION METHODS

3.1 Introduction

Great progress has been made in plant genetic engineering and numerous transformation methods have been developed in addition to *A. tumefaciens*-mediated transformation. Stable integration of introduced DNA has been achieved with isolated DNA fragments, total genomic DNA, chromosomes, and even isolated nuclei. This contrasts with transient gene transfer, in which the DNA is not integrated into the plant cell genome but is expressed for a relatively short time and subsequently lost. Virus-mediated gene transfer (CaMV, gemini virus), in which genes are maintained by extrachromosomal replication (Davies et al. 1987, Gronenborn 1987), should be placed in this category. The advantage of transient gene expression is the rapid assessment of gene expression achievable as a result of introducing

Table 1. Scientific milestones in plant genetic engineering

Year	Event
1907	Discovery of the causative agent of crown gall disease, <i>Agrobacterium tumefaciens</i> (Smith and Townsend 1907)
1930	Discovery of the causative agent of hairy root disease, <i>Agrobacterium rhizogenes</i> (Riker et al. 1930)
1974	Discovery of large plasmids in <i>A. tumefaciens</i> (Zaenen et al. 1974)
1975	Identification of Ti plasmids as being responsible for tumour induction in plants (Van Larebeke et al. 1975, Watson et al. 1975)
1977	Regeneration of <i>N. tabacum</i> plants from <i>A. rhizogenes</i> -transformed roots (Ackermann 1977) First isolation of a plant gene (large subunit of ribulose biphosphate carboxylase from chloroplast genomes) from <i>Zea mays</i> (Coen et al. 1977) Evidence of genetic transformation by the T-DNA of <i>A. tumefaciens</i> Ti plasmids in plant tissues (Chilton et al. 1977)
1981	Recovery of a genetically transformed plant (Wullem et al. 1981)
1983	Plant genetic engineering with a modified T-DNA (Barton et al. 1983) Plant genetic engineering with functional bacterial chimeric genes (Fraley et al. 1983, Herrera-Estrella et al. 1983)
1985	Plant chloroplast genetic transformation (De Block et al. 1985) Transfer of herbicide tolerance in transgenic <i>N. tabacum</i> (Comai et al. 1985) Presence of gene silencers and enhancers in plants (Timko et al. 1985)
1987	Site-directed gene transfer in plants (Wirtz et al. 1987)
1988	Regeneration of a transgenic monocot from protoplast electroporation (<i>Zea mays</i>) (Rhodes et al. 1988)

very high copy numbers into the host cells. This type of transient gene transfer will not be reviewed here.

A novel type of genetic transformation has been established by combining *Agrobacterium*-mediated gene transfer with virus infection. The genomes of some viruses have been cloned in the T-DNA of *A. tumefaciens* Ti plasmid vectors and transferred to plant cells by either viral or bacterial inoculation methods. It is only necessary for a few cells to be genetically transformed to achieve virus infection of the entire plant (Grimsley and Bisaro 1987). This can be considered an amplification of the transformed cell phenotypes, but the plants are not transgenic organisms.

There are two approaches to gene transformation. Direct gene transfer can be accomplished using methods that deliver DNA directly to the plant cell without any intermediates. Indirect gene transfer employs an intermediate organism, such as one of the plant pathogens *A. tumefaciens* and *A. rhizogenes*, to transmit foreign DNA to the plant genome. This DNA must be first introduced to the intermediate organism (*Agrobacterium*), which in turn delivers the DNA to the plant genome via the T-DNA.

3.2 Direct gene transformation methods

Direct transformation methods overcome host range limitations inherent in using *Agrobacterium* species. For monocotyledon transformation, in particular Graminae, this is important because these species fall outside the host range of *A. tumefaciens*. Most of these methods rely on protoplasts from which plant regeneration can be achieved. Except for efficient methods such as microinjection, selection for the introduced DNA with the help of a marker gene (such as resistance to antibiotics) is needed to recover transformed cells.

3.2.1 Polyethylene glycol or polyvinyl alcohol delivery

Polyethylene glycol (PEG) or polyvinyl alcohol (PVA) can be used to permeabilize the plant protoplast membrane to stimulate passive uptake into the plant cell of naked DNA or precipitated DNA (with calcium ions) (Freeman et al. 1984, Chen et al. 1987, Junker et al. 1987, Negrutiu et al. 1987). Factors such as the concentration of PEG or PVA and the concentration of vector and carrier DNA influence the rates of transformation

(Shillito et al. 1985). Stable transformation was obtained with *N. tabacum* (Kohler et al. 1987a), *Saccharum* spp. (Chen et al. 1987), *Triticum monococcum* (Lörz et al. 1985), *Vigna aconitifolia* (Kohler et al. 1987a, b), *Oryza sativa* (Uchimiya et al. 1986), *P. hybrida* (Kruger-Lebus and Potrykus 1987), *Lolium multiflorum* (Lörz et al. 1985), *B. campestris* (Paszowski et al. 1986), and *Nicotiana plumbaginifolia* (Negrutiu et al. 1987). Transformation frequencies were relatively low, on the order of 10^{-6} to 10^{-5} ; however, frequencies of 10^{-3} to 10^{-4} have been obtained after optimization of factors with *P. hybrida* (Kruger-Lebus and Potrykus 1987). Transient expression was obtained with *Zea mays*, *Hordeum vulgare*, *O. sativa*, *Secale cereale* (Junker et al. 1987), and *T. monococcum* (Weer and Lörz 1986). Transgenic plants have been obtained with *P. hybrida* (Kruger-Lebus and Potrykus 1987) and *Dactylis glomerata* (Horn et al. 1988).

3.2.2 Electroporation

Electroporation is used to increase the frequency of transformation of the PEG or PVA methods (Shillito et al. 1985, Potrykus et al. 1987). Subjecting protoplasts to a high voltage electric pulse in the presence of DNA and PEG enhances the passage of DNA into plant protoplasts and changes the permeability of the cell membrane (Shillito et al. 1985, Potrykus et al. 1987) (Figure 1).

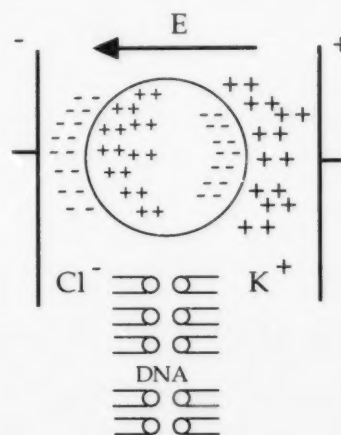


Figure 1. Electroporation of plant protoplasts. Protoplasts (circle) are submitted to an electric pulse (E) which permeabilizes the cell membrane. DNA enters passively through pores created by the current and the DNA can then be integrated into the plant cell genome by the host machinery.

Both stable integration (Langridge et al. 1985, Fromm et al. 1986, Bradshaw et al. 1987, Hauptmann et al. 1987, Rhodes et al. 1988) and transient expression have been achieved using this method (Ou-Lee et al. 1986, Bradshaw et al. 1987, Guerche et al. 1987a, Thornburg et al. 1987), and transformation frequencies are on the order of 10^{-3} to 10^{-4} . The use of protoplasts is a prerequisite, although this limits the number of plant species amenable to transformation by electroporation. Regeneration of transgenic plants from electroporated protoplasts has been successful with *Zea mays* (Rhodes et al. 1988), *N. tabacum* (Shillito et al. 1985, Tyagi et al. 1989), *O. sativa* (Toriyama et al. 1988, Zhang et al. 1988, Shimamoto 1989), *B. napus* (Guerche et al. 1987b), and *Lactuca sativa* (Chupeau et al. 1989).

3.2.3 Liposome-mediated delivery

In this approach, DNA is encapsulated in a phospholipid vesicle, the liposome, which is fused with plant protoplasts by PEG or cationic treatments (Fraley 1983, Caboche and Deshayes 1984, Deshayes et al. 1985, Rosenberg et al. 1988). Protection of nucleic acids from degradation by nucleases in the medium or the cytoplasm is the main advantage of liposomes. Transformation frequencies of 10^{-5} to 10^{-6} have been obtained (Fraley 1983, Caboche and Deshayes 1984, Deshayes et al. 1985). This method has been used successfully with *N. tabacum* protoplasts; transgenic plants were regenerated. Genetic analyses indicated that marker genes were transmitted to the progeny (Caboche and Deshayes 1984, Deshayes et al. 1985).

3.2.4 Spheroplast-mediated delivery

Fusion of plant protoplasts with spheroplasts prepared from *Escherichia coli* or *A. tumefaciens* was accomplished with PVA or PEG. DNA inside *E. coli* or *A. tumefaciens* was integrated into the protoplast genome. This method has been used to transform *Vinca rosea* (Hasezawa et al. 1981, 1983) with *E. coli* and *A. tumefaciens* (Matsui et al. 1983), *B. napus* with *E. coli* (Tanaka et al. 1984), *N. tabacum* with *A. tumefaciens* and *E. coli* (Hain et al. 1984), and *O. sativa* with *A. tumefaciens* spheroplasts (Baba et al. 1986). Transformation frequencies of 10^{-4} to 10^{-3} have been obtained (Hain et al. 1984, Baba et al. 1986), but no transgenic plants have been regenerated. A drawback of this method is the introduction of more DNA than is desirable. This could lead to

mutagenesis and recombination events in genes, to the detriment of the organism, as has been shown in bacterial transformation (Grist and Butler 1983).

3.2.5 Microinjection

Microinjection permits direct and precise delivery of DNA into the plant cell or its nucleus. The DNA can be introduced into the nucleus of isolated protoplasts (Crossway et al. 1986, Reich et al. 1986a, b, c, Miki et al. 1987) or into plant cells (Crossway et al. 1986, Neuhaus et al. 1987) using a microsyringe. For injection into the nucleus, dyes in combination with fluorescence microscopy (Reich et al. 1986a) or bright field microscopy (Crossway et al. 1986) can be used to visualize the nucleus. Protoplasts may be held with a holding pipette or embedded in a gelling agent. Precision of DNA delivery and extremely high transformation frequencies are the main advantages of this method. Transformation frequencies of 15% to 26% with *Medicago sativa*, 66% with *B. napus*, and 14% with *N. tabacum* were obtained by intranuclear microinjection of protoplasts (Crossway et al. 1986, Reich et al. 1986b, Miki et al. 1987). With cytoplasmic microinjection of *N. tabacum* protoplasts, 6% transformation was obtained (Crossway et al. 1986). Recently, microinjection of *B. napus* microspore-derived embryos gave transformation frequencies of 10 to 30% (Neuhaus et al. 1987). Transgenic plants were obtained with *N. tabacum* (Crossway et al. 1986, Neuhaus et al. 1987) and *B. napus* (Neuhaus et al. 1987). Microinjection is at present a tedious procedure and the equipment needed is expensive. Moreover, only a limited number of cells can be microinjected in one experiment.

3.2.6 Microprojection

Microprojection is a relatively new technique in which microprojectiles, made by coating small tungsten (Klein et al. 1987, 1988a,b,c) or gold (Christou et al. 1988, 1989) particles with DNA, are accelerated with a particle gun (air, gas, gun powder, or electrical) to velocities that permit penetration of intact cells (Figure 2; Sanford et al. 1987, Morikawa et al. 1989, Oard et al. 1990). Factors affecting transformation include the volume and size of the microprojectile, the amount of DNA, the presence of CaCl_2 and spermidine, and the vacuum strength (Klein et al. 1988a,b,c). Transient expression of transferred

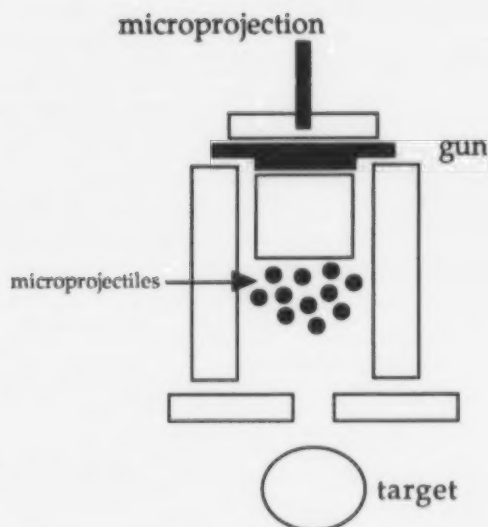


Figure 2. Microprojection of DNA into plant cells or tissues. Tungsten or gold microprojectiles are coated with DNA and projected using an electrical discharge, gun powder, or gas flow. The microprojectiles enter the tissues and the DNA can be integrated into the cell genome.

DNA has been shown in numerous plant species (*Oryza sativa*: Bruce et al. 1989, Oard et al. 1990, McElroy et al. 1990, and Wang et al. 1988; *Hordeum vulgare*: Mendel et al. 1989 and Kartha et al. 1989; *Zea mays*: Klein et al. 1988a,b,c, 1989a,b, Ludwig et al. 1990, and Oard et al. 1990; *Triticum aestivum*: Wang et al. 1988 and Oard et al. 1990; *L. esculentum*: Twell et al. 1989; and *Allium cepa*: Klein et al. 1987). Transgenic plants were obtained with *Glycine max* (McCabe et al. 1988, Christou et al. 1989), *N. tabacum* (Klein et al. 1988, Tomes et al. 1990), and *Gossypium hirsutum* (Finer and McMullen 1990). The use of intact cells and tissues with microprojection is a major advantage because it bypasses tissue culture steps. Chloroplast (Boynton et al. 1988) and mitochondrial (Johnston et al. 1988) transformations with microprojection have been accomplished in *Chlamydomonas reinhardtii* and *Saccharomyces cerevisiae*, respectively.

3.2.7 Macroinjection

Macroinjection involves the injection of DNA, using a syringe, into the floral parts of a plant. Successful transformation has been reported only once (De La Pena et al. 1987). Transgenic *Secale cereale* plants were obtained by injection of DNA (plasmid with NPT II gene) into young floral tillers, which are sensitive to colchicine or caffeine

treatment, suggesting that they were also permeable to other molecules such as DNA (Puertas et al. 1984). Transformation of seedlings from the macroinjected plants was verified by detection of the NPT II gene and resistance to kanamycin, but the frequency of transformation was relatively low (0.3%). The advantage of macroinjection, if proved to be repeatable, is that it bypasses the limitations of tissue culture procedures for recovering transgenic cereal plants.

3.2.8 Laser microbeam delivery

This method involves puncturing plant cells using an ultraviolet laser microbeam, which allows DNA to enter the cell (Weber et al. 1988, 1989a,b). Transient expression has been shown in microspore-derived embryos of *B. napus* (Weber et al. 1989b). No direct proof of stable transformation has been reported. Consequently, no transgenic plants have been regenerated. The advantage of such a method is that it allows the use of intact cells.

3.2.9 Pollen tube-mediated delivery

This method involves the transfer of exogenous DNA via the pollen tube pathway during its germination. This has been accomplished with *Oryza sativa*, where the stigma was cut off the floret and a solution of DNA deposited on top of the cut style (Duan and Chen 1985, Luo and Wu 1989). It has been suggested that the DNA reached the ovule by going down the pollen tube. Transgenic rice plants were regenerated from the progeny of the DNA-treated plants; however, the results have raised a lot of controversy and are disputed by certain groups.

3.2.10 Delivery through seed imbibition

This method has not yet given any final indication of stable transformation. It consists of imbibing seeds in an aqueous solution of DNA and allowing the DNA to enter the seeds. It has been tested with seeds of *Avena sativa*, *Hordeum vulgare*, *Oryza sativa*, *Triticum monococcum*, *Secale cereale*, *Zea mays*, *Phaseolus coccineus*, *Pisum sativum*, and *Vicia faba* and only transient gene expression has been observed (Topfer et al. 1989).

3.2.11 Silicon carbide fibre-mediated delivery

Microscopic silicon carbide fibres have been used to deliver exogenous DNA into cells of *Z. mays* and *N. tabacum* (Kaeppeler et al. 1990). The method is based on the microperforation of the cells following vortexing with the micro-fibres,

which allows entry of DNA and the subsequent expression of the genes introduced. No transgenic tissues have been recovered as yet.

3.3 Indirect gene transfer

Agrobacterium has the ability to transfer and integrate DNA into plant genomes through a unique interkingdom gene transfer mechanism. This property has been exploited for genetic engineering of plants with *A. tumefaciens* and *A. rhizogenes*. Both are phytopathogenic bacteria and members of the Rhizobiaceae. *A. tumefaciens* is the causative agent of crown gall disease and *A. rhizogenes* is responsible for hairy root disease (Lippincott et al. 1981). Both species induce diseases in a wide range of gymnosperms and dicotyledonous angiosperms (De Cleene and De Ley 1976, 1981) but monocotyledonous angiosperms are naturally resistant to *Agrobacterium* infection (De Cleene 1985).

3.3.1 Natural genetic transformation by *Agrobacterium*

The diseases are due to the large (150-250 kilobases) Ti plasmids present in *Agrobacterium*

and the root-inducing (Ri) plasmids in *A. rhizogenes* (Van Larebeke et al. 1975, Watson et al. 1975, White and Nester 1980a,b). These plasmids code for the disease characters and, when they are transferred from one *Agrobacterium* to another or even to *Rhizobium*, the ability to induce a specific disease is also transferred (Watson et al. 1975). Ti plasmids are classified into four categories: nopaline, octopine, agropine, and succinamopine plasmids (Petit et al. 1983, Nester et al. 1984, Eckes et al. 1987), and Ri plasmids into three categories: mannopine, agropine, and cucumopine plasmids (Biot et al. 1987) (Table 2). These compounds or opines are amino acid or sugar derivatives specifically produced by plant tumour tissues and they can be used by *Agrobacterium* as a sole source of carbon and nitrogen (Petit et al. 1983, Nester et al. 1984, Eckes et al. 1987).

3.3.1.a The role of T-DNA

Crown gall and hairy root diseases are caused by the expression of hormone synthesis genes (oncogenicity or onc genes) located on a portion of the Ti or Ri plasmids transferred and integrated

Table 2. Classification of Ti and Ri plasmids of *Agrobacterium*

Class	Opines synthesized	Example	Incompatibility group
<i>A. tumefaciens</i>			
Octopine	Octopine family + agropine family	pTiA6 pTiB6S3 pTiAch5	Rh-1
Nopaline	Nopaline family + agrocinopines A and B	pTiC58 pTiT37	Rh-1
Agropine	Agropine family + agrocinopines C and D	pTiB0542	Rh-2
Succinamopine	Succinamopine family	pTiAT181	?
<i>A. rhizogenes</i>			
Agropine	Agropine family + agrocinopines A and B	pRiA4	Rh-3
Mannopine	Agropine family (except agropine) + agrocinopines C + D	pRi8196	Rh-3
Cucumopine	Cucumopine	NCPPB2659	?

Note: The octopine family includes octopine, octopinic acid, lysopine, and histopine; the nopaline family includes nopaline and nopalinic acid; the succinamopine family includes succinamopine, succinamopine lactam, and succinopine lactam; and the agropine family includes agropine, mannopine, mannopinic acid and agropinic acid. ? = not determined.

into the plant genome (reviewed by Weiler and Schroder 1987, Spena et al. 1987, and Birot et al. 1987) and called T-DNA or transferred DNA (Chilton et al. 1977, 1982). Along with the hormone genes on the T-DNA are opine synthase genes that will be expressed in transformed tissues and produce specific opines corresponding to the Ti plasmid class (Nester et al. 1984, Eckes et al. 1987). A schematic of T-DNA is shown in Figure 3.

has been found to be positively affected by sequences close to the right border and negatively affected by sequences close to the left border (Wang et al. 1987a). Both studies favour the hypothesis of polar transfer of T-DNA to plant cells.

The most probable mechanism for T-DNA transfer involves single-stranded site specific nicking of the borders and the production of a

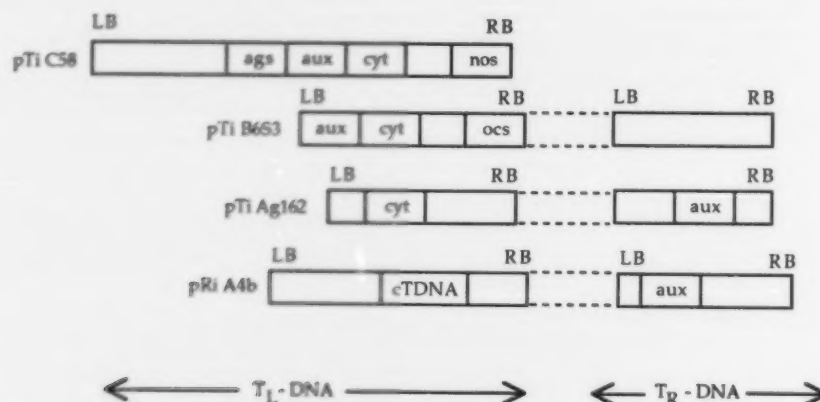


Figure 3. Structure of various T-DNA from Ti and Ri plasmids. T_L -DNA = left T-DNA, T_R -DNA = right T-DNA, LB = left border, RB = right border. Hormone synthesis genes: aux = auxin synthesis, cyt = cytokinin synthesis. Opine synthesis genes: ags = agropine synthesis, nos = nopaline synthesis, ocs = octopine synthesis. cDNA = DNA hybridizing with plant DNA. Adapted from Nester et al. (1984).

Hormone genes of the T-DNA of Ri and Ti plasmids have been isolated and characterized. They encode enzymes involved in the biosynthetic pathways of auxins and cytokinins (Weiler and Schroder 1987). These genes have been used in genetic transformation experiments to alter the growth regulator balance of the plant tissue, thus changing their growth characteristics (Medford et al. 1989).

The genes inside the T-DNA can be deleted with no effect on processing, transfer, and integration. However, two 25-basepair repeats at the border of all T-DNAs are needed for their efficient transfer. The right border has been found to be essential, whereas the left border can be deleted and T-DNA transfer will still occur (Peralta and Ream 1985, Peralta et al. 1986, Wang et al. 1987a). In octopine plasmids, such as pTiA6NC, a 23-basepair sequence next to the right border (the overdrive) was found to stimulate T-DNA transmission (Peralta et al. 1986). Furthermore, T-DNA transfer in nopaline pTiC58

single-stranded T-DNA (T-strand) which is produced by displacement of the old s'rand (Yanofsky and Nester 1986, Yanofsky et al. 1986, Stachel et al. 1987, Wang et al. 1987b, Christie et al. 1988, Veluthambi et al. 1988). An alternative view is the double-stranded site specific nicking of the right border and the generation of a circular or linear double-stranded T-DNA (Koukolikova-Nicola et al. 1985, Alt-Moerbe et al. 1986, Jayaswal et al. 1987, Veluthambi et al. 1987, Yamamoto et al. 1987). However, it is possible that such molecules are generated by the experimental procedures and may not be involved in T-DNA transfer to the plant cell. Moreover, the frequency of such events is relatively low (Koukolikova-Nicola et al. 1985).

Some hormone genes on the T-DNA are also important in controlling the host range of *A. tumefaciens* (Yanofsky et al. 1985a,b). Their actions are at the tumour-formation level and not at the transformation stage.

It is important to note that T-DNA genes have evolved to be expressed in the plant cell and not in bacteria. The hormone imbalance caused by T-DNA genes in plant tissues will induce disease; tumour formation in the case of Ti plasmids and hairy roots in the case of Ri plasmids. The opines produced in the tumour or the root allow for colonization of plant tissues. The opines also induce *Agrobacterium* mating and the transfer of Ti or Ri plasmids.

3.3.1.b The role of the VIR region

Another region, called the virulence or VIR region, of about 35 kilobases on Ti or Ri plasmids is essential for the transfer of T-DNA to the plant genome. It acts *in trans* (from one plasmid onto another plasmid) to the T-DNA (Hoekema et al. 1983). The genes of the VIR region of Ti or Ri plasmids are responsible for the processing of the T-DNA and its transfer to the plant cell. They are also involved in host specificity of Ti or Ri plasmids (Loper and Kado 1979, Thomashow et al. 1980, Knauf et al. 1984). Six VIR operons (VIR A,B,C,D,E, and G) have been identified in both octopine (Stachel and Nester 1986) and nopaline (Rogowsky et al. 1987) Ti plasmids. Homology between VIR regions of octopine and nopaline Ti plasmids and Ri plasmids has been found (Risuelo et al. 1982, Jouanin 1984, Knauf et al. 1984, Janssens et al. 1986, Rogowsky et al. 1987). In octopine Ti plasmids, a seventh operon, VIR F, has been identified that does not have any counterparts in other Ti plasmid types (Stachel and Nester 1986). In nopaline Ti plasmids, the VIR region contains the locus *tzs*, which encodes an enzyme for synthesizing transzeatin in *Agrobacterium* (Janssens et al. 1986). The VIR F and *tzs* loci are induced by plant exudates.

VIR A,B,D, and G operons are essential for tumour induction. VIR C,E, and F operons are necessary only with certain plant species (Stachel and Nester 1986, Rogowsky et al. 1987). VIR A and G operons are expressed constitutively in *Agrobacterium*, but the VIR G operon can be induced under specific conditions, such as in the presence of plant phenolic compounds synthesized at the wound site, through phosphate starvation, and in acidic media (Winans 1990). VIR B,C,D,E, and F operons are induced by co-cultivation of *Agrobacterium* with plant cells or by incubation with plant phenolics such as acetosyringone or hydroxyacetosyringone (Stachel et al. 1985a, 1986a, Stachel and Nester 1986,

Rogowsky et al. 1987). Certain opines (octopine, nopaline, leucinopine, and succinamopine) are inducers of VIR genes in combination with acetosyringone (Veluthambi et al. 1989).

The roles of VIR genes have not been completely elucidated, but strong experimental evidence suggests possible functions for most of them. The VIR A operon, which contains one open reading frame (ORF) (Melchers et al. 1987), is thought to code for an inner membrane sensory protein for plant signal molecules (Stachel and Nester 1986, Melchers et al. 1987, Rogowsky et al. 1987). The VIR A protein would, in turn, directly or indirectly activate the VIR G protein product. This last operon comprises one ORF that codes for a positive regulatory protein activating the other VIR genes (Stachel and Nester 1986, Winans et al. 1986, Rogowsky et al. 1987). VIR A and VIR G affect host-range specificity (Jin et al. 1987, Leroux et al. 1987).

Other VIR operons are more complex and only a few proteins have been assigned potential roles. The VIR D operon possesses five ORFs (Porter et al. 1987). VIR D1 and VIR D2 proteins are thought to be part of a site-specific endonuclease that cleaves the bottom strand of the T-DNA at the borders (Alt-Moerbe et al. 1986, Stachel et al. 1986b, 1987, Yanofsky et al. 1986, Jayaswal et al. 1987, Porter et al. 1987, Howard et al. 1989), generating the T-strand molecule in *Agrobacterium* by displacement. The VIR D gene encodes a DNA-relaxing enzyme required for cleaving the single-stranded T-DNA (Ghai and Das 1989). The VIR E operon includes two ORFs in pTiA6 and at least five ORFs in pTiC58 (Hirooka et al. 1987, McBride and Knauf 1988). The VIR E2 gene product has been found to bind to single-stranded DNA and is located in the cytoplasm, the inner and outer membranes, and the periplasmic fractions of acetosyringone-induced *A. tumefaciens* (Christie et al. 1988, Das 1988, Sen et al. 1989). The VIR E2 protein is thought to be involved in processing the T-DNA and in T-strand protection (Christie et al. 1988). The VIR E operon has a role in host-range specificity (Hirooka and Kado 1986, McBride and Knauf 1988). However, VIR E is not strictly required for tumour formation and can be complemented by co-infection with an *A. tumefaciens* strain containing a nondefective VIR E operon (Gardner and Knauf 1986).

The VIR B operon includes 11 ORFs (Ward et al. 1988). There is some evidence of membrane association of VIR B gene products, suggesting that they would be involved in the formation of a transmembrane complex for the delivery of the T-DNA to the plant cell. VIR B1 to VIR B9 operons are localized in the cell envelope (Thompson et al. 1988). VIR B4 and VIR B11 also contain sequences needed for binding ATP, which supports the idea of a delivery complex for T-DNA (Thompson et al. 1988). The VIR B operon influences the host-range specificity of *A. tumefaciens* (Jin et al. 1987).

The VIR C locus contains two ORFs (Yanofsky and Nester 1986) and it affects host range (Srinivasan et al. 1989). It also elicits a hypersensitive response in *Vitis* spp. and renders the plant resistant to tumour induction by *A. tumefaciens* (Yanofsky and Nester 1986).

Table 3 presents a summary of VIR gene functions.

Table 3. Summary of possible functions for chromosomal and virulence genes involved in *Agrobacterium* transformation

Functions		
Chromosomal genes		
<i>chv A, chv B, att, exoC</i>		Attachment to plant cell
<i>ros, chv D, chv E</i>		VIR gene regulation
"chemotaxis"		<i>Agrobacterium</i> chemotaxis
Virulence genes		
VIR A		Chemoreceptor, activator of VIR
VIR B		Transmembrane complex
VIR C		Host-range specificity
VIR D		Site-specific endonuclease and DNA-relaxing enzyme
VIR E		T-DNA processing and protection
VIR F		Host-range specificity
VIR G		Positive regulator of VIR B, C, D, E, F, and <i>tzs</i>

3.3.1.c The role of *Agrobacterium* chromosomal genes

Tumour induction on plants by *Agrobacterium* is dependent on multiple factors. The host plant has to be susceptible to *Agrobacterium*, which in turn has to be virulent on the host. The host range of *Agrobacterium* is dependent upon the expression of the T-DNA hormone gene in the transformed cell, the virulence region of the Ti or Ri plasmids, and the chromosomal genes of *Agrobacterium*. The steps that are under the control of the bacterial chromosome (Matthysse et al. 1981, Matthysse

1983) are the loose and reversible site-specific attachment of *Agrobacterium* to the plant host cell, followed by the synthesis of cellulose fibrils by *Agrobacterium* to cover the plant cell surface. This step is irreversible and *Agrobacterium* is tightly bound to the host cell. Finally, clusters of *Agrobacterium* are entrapped in a cellulose fibril web.

The chromosomal genes involved in these steps have been identified and mapped by transposon mutagenesis. The genes *chvA* and *chvB* (Douglas et al. 1985) are involved in the synthesis of cellulose fibrils and mutants in these loci are defective in tight plant attachment. The *chvB* locus encodes the synthesis of β -1,2-glucan that is found in periplasmic and extracellular spaces (Zorreguieta et al. 1988). The motility of *Agrobacterium* and the ability to transfer the Ti plasmid in conjugation is affected by these genes. Two other genes are also involved in *Agrobacterium* attachment to the plant. The gene *Att* is associated with the synthesis of polypeptides involved in *Agrobacterium* spheroplast formation (Matthysse 1987, Robertson et al. 1988). Mutants of this gene fail to attach to plant cells. The last gene is *exoC* (or *pseA*) and it encodes the synthesis of succinoglycan needed for the synthesis of cellulose fibrils (Cangelosi et al. 1987, Thomashow et al. 1987).

Chromosomal genes are also involved in functions other than *Agrobacterium* attachment to the plant cell. The gene *ros* regulates the expression of VIR genes (Close et al. 1985). This locus acts as a negative regulator of VIR genes and mutants have VIR expression levels that are elevated 300-fold. *Agrobacterium* colony morphology is also affected. The genes *chvE* and *chvD* are positive regulators of VIR genes. Their inactivation by transposon mutagenesis reduces the virulence of *Agrobacterium* (Huang et al. 1990). Other chromosomal genes are responsible for *Agrobacterium* chemotaxis toward the plant, but these genes have not been mapped or characterized (Ashby et al. 1987, Parke et al. 1987, Loake et al. 1988). The chemotaxis system is based on the attraction of bacteria to chemicals, such as sugars, amino acids (Loake et al. 1988), and phenolics, that are released from the plant (Parke et al. 1987). Phenolics, such as acetosyringone and hydroxyacetosyringone, also act as chemoattractants involved in the mechanism encoded by the Ti plasmid and, apparently, by the

VIR A and G genes (Ashby et al. 1987, 1988, Shaw et al. 1988).

The Ti and Ri plasmids also contain bacterial genes involved in opine catabolism, conjugal transfer of the plasmids, replication functions, agrocin sensitivity, and phage exclusion (Nester and Kosuge 1981).

The *A. tumefaciens* genetic transformation process via the Ti plasmid is illustrated in Figure 4.

3.3.2 Genetic engineering of plants with *Agrobacterium* Ti or Ri plasmids

Ti and Ri plasmids have been used as vectors for plant genetic engineering. T-DNA of Ti plasmids have been disarmed by replacing the oncogenic functions (or hormone genes) with other DNA fragments (Zambryski et al. 1983). This allows the regeneration of phenotypically normal transgenic plants or tissues. Table 4 provides a list of disarmed Ti plasmids and Figures 5 and 6

illustrate the results of the disarming process. No disarmed Ri plasmid is available.

The first type of disarmed Ti plasmid was engineered by exchanging the oncogenic genes with pBR322 sequences via homologous gene recombination techniques (Van Haute et al. 1983, Zambryski et al. 1983). Both T-DNA borders were retained in this vector and the DNA to be transferred to the plant was introduced between them through homologous recombination with

Table 4. Engineered disarmed Ti plasmids and their origin

Disarmed Ti plasmid	Original Ti plasmid	Type	Reference
pGV3850	pTiC58	Cointegrate	Zambryski et al. (1983)
pGV2260	pTiB6S3	Cointegrate	Deblaere et al. (1985)
pMP90	pTiC58	Binary	Koncz and Schell (1986)
pAL4404	pTiAch5	Binary	Hoekema et al. (1983)
pEHA1001	pTiBo542	binary	Hood et al. (1986)
pTiB6S3-SE	pTiB6S3	Cointegrate	Fraley et al. (1985)
pTiB6S3-NE	pTiB6S3	Binary	Rogers and Klee (1987)
pTiT37-SE	pTiT37	Cointegrate	Rogers and Klee (1987)

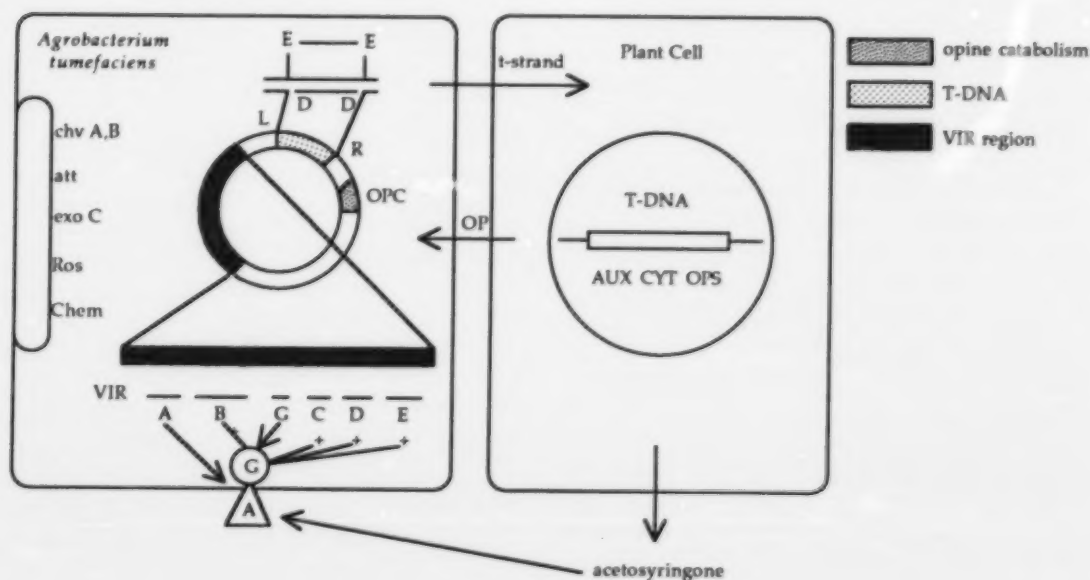


Figure 4. Schematic of molecular events involved in T-DNA transfer. The chromosomal genes involved in genetic transformation are *chvA,B*, *att*, *exoC*, *ros*, and *chem*. The VIR genes are A, B, G, C, D, and E and they are induced by chemicals, such as acetosyringone, through the chemoreceptor (VIR A gene products), which in turn activates the VIR G gene products. These then activate the other VIR genes. VIR D gene products are involved in T-DNA processing (L = left border, R = right border) and VIR E gene products in the transfer process. VIR B gene products are involved in a transmembrane complex for the transfer of the linear T-DNA. Once the T-DNA is integrated into the plant genome, the hormone genes (*aux*, *cyt*) and opine synthase genes (*ops*) are expressed in the plant cell.

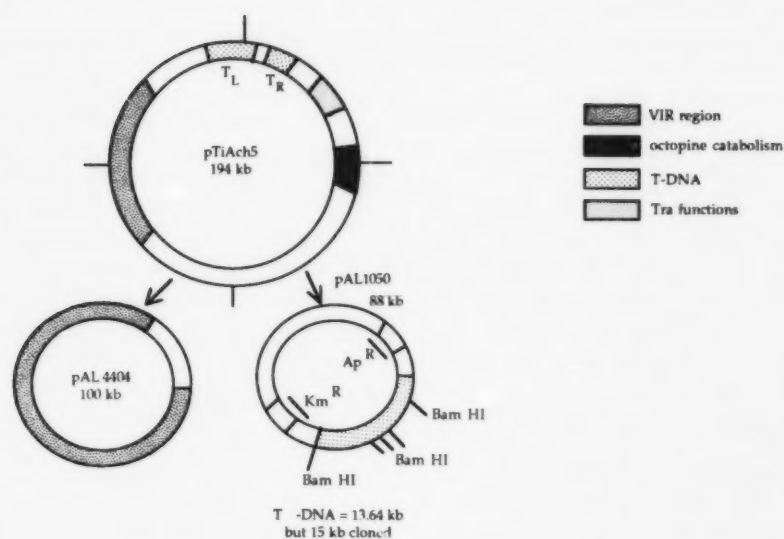


Figure 5. Derivation of disarmed Ti plasmid pAL4404 and its T-DNA pAL1050. The octopine plasmid pTiAch5 was mutated using transposons and a deletion mutant without T-DNA (pAL4404) was obtained (Ooms et al. 1982). The vector pAL4404 can only be used as a helper plasmid for binary vectors with their own T-DNA. The TL-DNA of pAL1050 was obtained by cloning into an intermediate vector, resulting in a binary vector (Hoekema et al. 1983). Abbreviations: kb = kilobases, Km = kanamycin, Ap = ampicillin, R = resistant, and Tra = transfer.

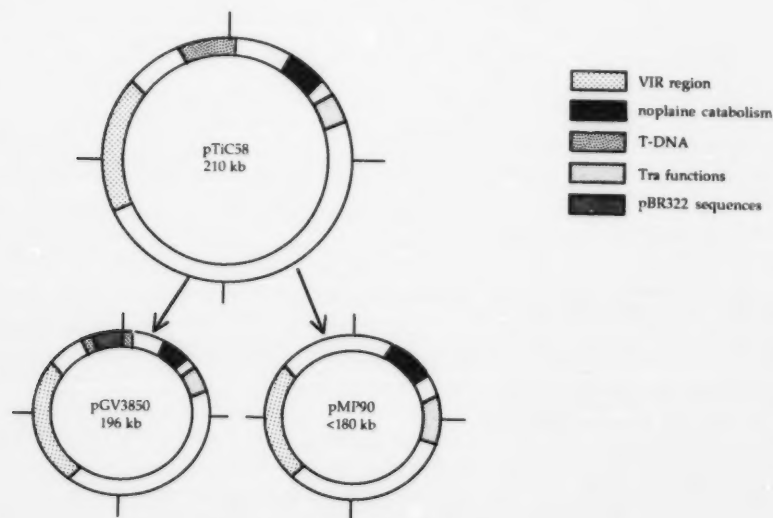


Figure 6. Derivation of disarmed Ti plasmids pGV3850 and pMP90. The nopaline Ti plasmid C58 was disarmed by a double recombination event with pBR322 (for pGV3850; Zambryski et al. (1983)) and with pTiD37 (for pMP90; Koncz and Schell (1986)). The plasmid pGV3850 can be used for cointegration in its pBR322 sequences, which are now the T-DNA sequences, or with a binary vector as a helper plasmid. The plasmid pMP90 can only be used as a helper plasmid for binary vectors with their own T-DNA. Abbreviations: kb = kilobases, and Tra = transfer functions.

pBR322 sequences. The genes were cloned and manipulated in *E. coli* and transferred by mating with *A. tumefaciens*. For this purpose, intermediate vectors with a narrow host range replicon were used because they could not replicate autonomously in *A. tumefaciens*. To be maintained, co-integration with the Ti plasmid is needed and antibiotic resistance selection pressure on the intermediate plasmid ensures the stability of the integration.

In most cases, a third plasmid was needed to mobilize the intermediate plasmid from *E. coli* to *Agrobacterium*. This plasmid, called a helper plasmid, is normally present in a second *E. coli* strain. Triparental mating is performed by mixing two *E. coli* strains and the *Agrobacterium* strain together and selection is applied to rescue strains in which co-integration of the intermediate plasmid vector with the Ti plasmid vector has occurred (Van Haute et al. 1983).

A second type of disarmed Ti plasmid has been constructed without the T-DNA borders (Hoekema et al. 1983, Hood et al. 1986, Koncz and Schell 1986). They can only be used *in trans* to another vector containing the T-DNA borders. These have been constructed with a wide host range replicon, which allows their autonomous replication in *Agrobacterium*. The T-DNA borders permit processing by VIR functions carried *in trans* either by disarmed or wild type Ti plasmids or Ri plasmids. This type of plasmid is called a binary vector because two different plasmids are needed in *Agrobacterium* for T-DNA transfer (Hoekema et al. 1983, Bevan 1984). The binary vector is manipulated in *E. coli* and transferred to *Agrobacterium* by mating, as described earlier. Antibiotic selection pressure is also needed to maintain the plasmid in *Agrobacterium* (Van Haute et al. 1983, Bevan 1984). The process of triparental mating is illustrated in Figure 7.

3.4 Recovery of transgenic plants

Transgenic plants have been recovered using direct and indirect gene transfer as described previously. To evaluate the status of this technology, a list of plant species that have been genetically engineered was compiled from the literature (Table 5). This list illustrates the severe constraints imposed by tissue culture systems on the development of technologies for producing transgenic plants.

3.4.1 Genetically transformed plant species

Transgenic plants have been obtained from 46 plant species, including dicots, monocots, and trees (Table 6). About one fifth of these species (11) belong to the Solanaceae family. In the Cruciferae, transgenic *B. napus* has been the subject of many transformation reports. Most of the work published outside these two families consists of single reports, indicating that the application of transformation technologies is still in the early stages. Monocots have been extremely difficult to engineer due both to the lack of regenerable tissue culture systems and recalcitrance to *Agrobacterium* transformation. Monocot transformation has been the focus of major research efforts worldwide, but so far only a few successes have been reported.

Agrobacterium-mediated transformation has been the most extensively developed method for plant genetic engineering of dicots. The neomycin phosphotransferase II (NPT II) gene has been a very useful marker for recovering transgenic tissues in monocots, dicots, and trees. For monocots, methods other than *Agrobacterium*-mediated gene transfer have been used with some success.

3.4.2 Genetic transformation markers and gene construction

Two general strategies can be followed to recover transgenic plants after protoplast, cell, tissue, or explant transformations. The first approach is to screen all of the plants that have been regenerated from a transformation experiment for the expression of a screenable (or reporter) gene. The second approach is to select the transformed protoplasts, cells, tissues, or explants for resistance to a selective agent, which is conferred by a selectable gene marker. Both types of marker genes are chimeric constructions that contain all of the signals needed for expression in the plant, but they may not be of plant origin (see next paragraph for a discussion on gene construction). Table 7 lists marker genes used in plant genetic engineering experiments, and their origin.

Reporter gene markers have a gene product or enzyme activity that can be easily detected. Very sensitive screening procedures have been

Table 5. Plant species from which transgenic plants have been recovered

Species	Common name	Methods	Marker	References
DICOTYLEDONS				
Solanaceae				
<i>Lycopersicon esculentum</i>	Tomato	<i>A. tumefaciens</i>	NPT II	McCormick et al. (1986), Bino et al. (1987)
		<i>A. rhizogenes</i>	NPT II	Shahin et al. (1986b)
<i>Lycopersicon peruvianum</i>		<i>A. rhizogenes</i>	H.R.	Morgan et al. (1987)
<i>Nicotiana clevelandii</i>		<i>A. tumefaciens</i>	NPT II	Tavazza et al. (1988)
<i>Nicotiana edwardsonii</i>		<i>A. tumefaciens</i>	NPT II	Kiernan et al. (1989)
<i>Nicotiana glauca</i>		<i>A. rhizogenes</i>	NPT II	Morgan et al. (1987)
<i>Nicotiana plumbaginifolia</i>		<i>A. tumefaciens</i>	Bleo	Hille et al. (1986)
		<i>A. tumefaciens</i>	NPT II	Trinh et al. (1987)
<i>Nicotiana sylvestris</i>		<i>A. tumefaciens</i>	NPT II	Neuhaus et al. (1991)
<i>Nicotiana tabacum</i>	Tobacco	<i>A. tumefaciens</i>	NPT II	Horsch et al. (1985)
		<i>A. tumefaciens</i>	SPT	Jones et al. (1987)
		<i>A. tumefaciens</i>	HPT	Waldron et al. (1985)
		<i>A. tumefaciens</i>	CAT	De Block et al. (1985)
		Electroporation	NPT II	Paszkowski et al. (1984)
<i>Petunia hybrida</i>	Petunia	<i>A. tumefaciens</i>	NPT II	Wallroth et al. (1986), Meyer et al. (1987), Sanders et al. (1987)
		<i>A. tumefaciens</i>		Eichholtz et al. (1987)
<i>Solanum melongena</i>	Eggplant	<i>A. tumefaciens</i>	NPT II	Guri and Sink (1988), Filipone and Lurquin (1989)
<i>Solanum nigrum</i>	Black nightshade	<i>A. rhizogenes</i>	H.R.	Wei et al. (1986)
<i>Solanum tuberosum</i>	Potato	<i>A. tumefaciens</i>	NPT II	Shahin and Simpson (1986), Ooms et al. (1987), Eckes et al. (1986), Hanisch et al. (1988)
Leguminosae				
<i>Glycine canescens</i>	Wild soybean	<i>A. rhizogenes</i>	H.R.	Rech et al. (1988)
<i>Glycine max</i>	Soybean	<i>A. tumefaciens</i>	NPT II	Hinchee et al. (1988)
		Microprojection	NPT II	McCabe et al. (1988)
<i>Lotus corniculatus</i>	Trefoil	<i>A. rhizogenes</i>	H.R., CAT	Stougaard-Jensen et al. (1986) Webb et al. (1990)
<i>Medicago sativa</i>	Alfalfa	<i>A. tumefaciens</i>	NPT II	Shahin et al. (1986a)
<i>Medicago varia</i>	Alfalfa	<i>A. tumefaciens</i>	NPT II	Deak et al. (1986)
<i>Pisum sativum</i>	Pea	<i>A. tumefaciens</i>	HPT	Puonti-Kaerlas et al. (1990)
<i>Stylosanthes</i> spp.	Townsville stylo	<i>A. tumefaciens</i>	NPT II	Manners (1988)
<i>Trifolium repens</i>	White clover	<i>A. tumefaciens</i>	NPT II	White and Greenwood (1987)
Cruciferae				
<i>Arabidopsis thaliana</i>		<i>A. tumefaciens</i>	HPT	Lloyd et al. (1986)
		<i>A. tumefaciens</i>	NPT II	Feldmann and Marks (1987)
<i>Brassica juncea</i>	Mustard	<i>A. tumefaciens</i>	NPT II	Mathews et al. (1990b)
<i>Brassica napus</i>	Rapeseed	<i>A. tumefaciens</i>	NPT II	Charest et al. (1988)
		<i>A. tumefaciens</i>	CAT	
		<i>A. tumefaciens</i>	DHFR	Pua et al. (1987)
		<i>A. tumefaciens</i>	NPT II	Fry et al. (1987)
		Electroporation	NPT II	Guerche et al. (1987b)
		Microinjection	NPT II	Neuhaus et al. (1987)
		<i>A. rhizogenes</i>	H.R.	Guerche et al. (1987c)
<i>Brassica oleracea</i>	Cauliflower	<i>A. rhizogenes</i>	H.R.	David and Tempe (1988)

Table 5. (continued)

Species	Common name	Methods	Marker	References
<i>Linum usitatissimum</i>	Flax	<i>A. tumefaciens</i>	NPT II	Basiran et al. (1987), Jordan and McHughen (1988)
Other dicotyledons				
<i>Apium graveolens</i>	Celery	<i>A. tumefaciens</i>	NPT II	Catlin et al. (1988)
<i>Armoracia lapathifolia</i>	Horseradish	<i>A. rhizogenes</i>	H.R.	Noda et al. (1987)
<i>Atropa belladonna</i>	Belladonna	<i>A. tumefaciens</i>	NPT II	Mathews et al. (1990a)
<i>Beta vulgaris</i>	Sugar beet	<i>A. tumefaciens</i>	NPT II CAT GUS	Lindsay and Gallois (1990)
<i>Catharanthus roseus</i>		<i>A. rhizogenes</i>	H.R.	Brillianceau et al. (1989)
<i>Convolvulus arvensis</i>	Morning glory	<i>A. rhizogenes</i>	H.R.	Slightom et al. (1985)
<i>Cucumis melo</i>	Muskmelon	<i>A. tumefaciens</i>	NPT II	Fang and Grumet (1990)
<i>Cucumis sativus</i>	Cucumber	<i>A. rhizogenes</i> <i>A. tumefaciens</i>	NPT II NPT II	Trulson et al. (1986) Chee (1990)
<i>Daucus carota</i>	Carrot	<i>A. rhizogenes</i>	H.R.	David et al. (1984)
<i>Fragaria x ananassa</i>	Strawberry	<i>A. tumefaciens</i>	NPT II	Nehra et al. (1990)
<i>Gossypium hirsutum</i>	Cotton	<i>A. tumefaciens</i>	NPT II, CAT	Firoozabady et al. (1987), Umbeck et al. (1987)
<i>Helianthus annuus</i>	Sunflower	<i>A. tumefaciens</i>	NPT II	Everett et al. (1987)
<i>Kalanchoe laciniata</i>		<i>A. tumefaciens</i>	NPT II	Jia et al. (1989)
<i>Lactuca sativa</i>	Lettuce	<i>A. tumefaciens</i> Electroporation	NPT II NPT II	Michelmores et al. (1987) Chupeau et al. (1989)
<i>Ribes nigrum</i>	Black currant	<i>A. tumefaciens</i>	NPT II GUS	Graham and McNicol 1991
MONOCOTYLEDONS				
<i>Asparagus officinalis</i>	Asparagus	<i>A. tumefaciens</i>	NPT II	Bytebier et al. (1987)
<i>Dactylis glomerata</i>	Orchard grass	Electroporation	HPT	Horn et al. (1988)
<i>Oryza sativa</i>	Rice	Electroporation	NPT II	Toriyama et al. (1988), Shimamoto et al. (1989)
<i>Secale cereale</i>	Rye	Macroinjection	NPT II	De La Pena et al. (1987)
<i>Zea mays</i>	Corn	Electroporation	NPT II	Rhodes et al. (1988)
<i>Zea mays</i>	Corn	Microprojection	PAT	Gordon-Kamm et al. (1990)
TREES (Dicots)				
<i>Allocasuarina verticillata</i>		<i>A. rhizogenes</i>	H.R.	Phelep et al. (1991)
<i>Azadirachta indica</i>	Neem	<i>A. tumefaciens</i>	NPT II	Naina et al. (1989)
<i>Citrus jambhiri</i>	Rough lemon	PEG delivery	NPT II	Vardi et al. (1990)
<i>Juglans regia</i>	Walnut	<i>A. tumefaciens</i>	NPT II	McGranahan et al. (1988)
<i>Malus pumila</i>	Apple tree	<i>A. tumefaciens</i>	NPT II	James et al. (1989)
<i>Populus alba x grandidentata</i>	Poplar	<i>A. tumefaciens</i>	NPT II EPSP	Fillatti et al. (1987b)
<i>Vitis rupestris</i>	Grapevine	<i>A. tumefaciens</i>	NPT II GUS	Mullins et al. (1990)

Note: This list includes all the transgenic plant species that have been reported in the literature. In plant species where normal transgenic plants were obtained, transgenic plants obtained through *A. rhizogenes* are omitted. Abbreviations: NPT II = neomycin phosphotransferase II, H.R. = hairy root, Bleo = bleomycine resistance, SPT = spectinomycin phosphotransferase, HPT = hygromycin phosphotransferase, CAT = chloramphenicol acetyltransferase, DHFR = dihydrofolate reductase, EPSP = 5-enolpyruvylshikimate-3-phosphate synthase, and PAT = phosphinotricin acetyltransferase, GUS = β -glucuronidase.

Table 6. Total number of species from which plants and transformed calli have been recovered

	Transgenic plants recovered	
	Normal phenotype	Hairy root phenotype
Dicotyledons		
Solanaceae	9	3
Leguminosae	7	1
Cruciferae	4	1
Others	12	5
Tree (dicots)	8	1
Monocotyledons	5	
Total	45	11

developed for the nopaline synthase gene (Holbrook et al. 1986), the chloramphenicol acetyltransferase gene (De Block et al. 1984), the β -glucuronidase gene (Jefferson et al. 1987), and luciferase genes (Ow et al. 1986, Koncz et al. 1987). All of these genes can be detected by the activity

of the enzymes they encode. The β -glucuronidase and luciferase genes can be visualized *in situ* by their end products (Ow et al. 1986, Jefferson et al. 1987, Koncz et al. 1987). Expression of β -galactosidase and luciferase genes can be detected by incubating the transformed plant tissues with their respective substrates without destroying the tissues tested (Helmer et al. 1984, Ow et al. 1986, Koncz et al. 1987).

Selectable gene markers are used to discriminate transformed cells from nontransformed cells. Generally, selectable markers are genes that will detoxify an antibiotic or an antimetabolite added to the culture medium. An exception to this rule is dihydrofolate reductase, a mutant form of a similar enzyme present in plants resistant to the drug methotrexate (Eichholtz et al. 1987). The

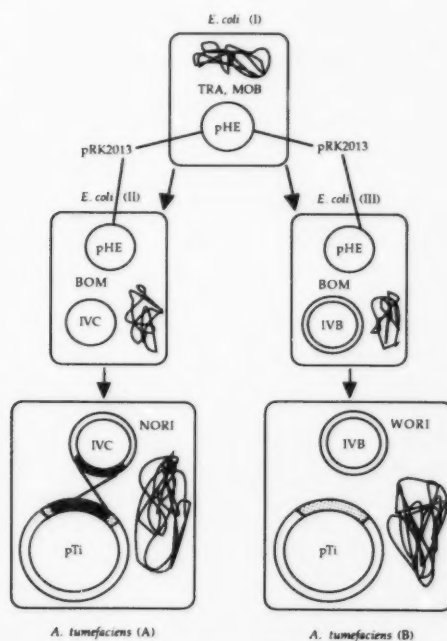


Figure 7. Triparental mating between *E. coli* and *A. tumefaciens*. The binary (IVB) on *E. coli* III or cointegrating (IVC) vectors in *E. coli* (*E. coli* II) are mobilized with assistance of a helper plasmid (pHE) in another *E. coli* (*E. coli* I) strain that possesses the transfer (TRA) and mobilization (MOB) genes. Their gene products act *in trans* on the bacterial origin of mobilization (BOM) sites of the intermediate vectors. These are transferred to *A. tumefaciens*. The intermediate vector of the cointegrating type has a narrow host range origin of replication (NORI) and must be integrated into the Ti plasmid for stable maintenance (*A. tumefaciens* A). The intermediate vector of the binary vector has a wide host range origin of replication (WOR) and can be autonomously maintained in *A. tumefaciens* (B).

Table 7. Marker genes used in plant genetic engineering

Screenable marker	Source	Substrate	Reference
nopaline synthase	Ti plasmid	¹⁴ C-arginine	Holbrook et al. (1986)
octopine synthase	Ti plasmid	¹⁴ C-arginine	Holbrook et al. (1986)
chloramphenicol acetyltransferase	Tn9	¹⁴ C-chloramphenicol	De Block et al. (1984)
neomycin phosphotransferase	Tn5	γ - ³² P-ATP	Herrera-Estrella et al. (1983)
luciferase	<i>Photenus pyralis</i>	luciferine	Ow et al. (1986)
luciferase	<i>Vibrio harveyi</i>	decanal	Koncz et al. (1987)
β -glucuronidase	<i>E. coli</i>	X-glu, MUG, PNPG	Jefferson et al. (1987)
β -galactosidase	<i>E. coli</i>	X-gal, ONPG	Helmer et al. (1984)
Selectable marker	Source	Resistance	Reference
chloramphenicol acetyltransferase	Tn9	chloramphenicol	De Block et al. (1984)
neomycin phosphotransferase I	Tn601	kanamycin	Fraley et al. (1983)
neomycin phosphotransferase II	Tn5	kanamycin, geneticin, paromomycin	Fraley et al. (1983), Herrera-Estrella et al. (1983), Guerche et al. (1987b)
hygromycin phosphotransferase	<i>E. coli</i>	hygromycin	Waldron et al. (1985)
dihydrofolate reductase	pR67 Mouse	methotrexate methotrexate	Herrera-Estrella et al. (1983) Eichholtz et al. (1987)
streptomycin phosphotransferase	Tn5	streptomycin	Jones et al. (1987)
Bleomycin resistance	Tn5	bleomycin, phleomycin	Hille et al. (1986), Perez et al. (1989)
gentamicin acetyltransferase	pLG62	gentamicin, kanamycin	Hayford et al. (1988)
Bleomycin resistance	<i>Streptoalloteichus hindustanus</i>	bleomycin	Perez et al. (1989)
Phleomycin resistance	<i>Streptoalloteichus hindustanus</i>	phleomycin	Perez et al. (1989)
dihydropteroate synthase	pR46	Sulfonamides	Guerineau et al. (1990)

Abbreviations: X-glu = 5-bromo-4-chloro-3-indolyl glucuronide, MUG = 4-methyl umbelliferyl glucuronide, PNPG = p-nitrophenol glucuronide, X-gal = 5-bromo-4-chloro-3-indolyl galactoside, and ONPG = 0-nitrophenyl-b-D-galactopyranoside.

advantage of selection is to reduce the amount of nontransformed material to be handled. In certain plant tissue culture systems, where transformants are very infrequent, the selective agent is ineffective in the recovery of transformed cells because they may be sensitive to the metabolites released by the surrounding dying nontransformed cells. Neomycin phosphotransferase II has been the most widely used selectable marker because it is considered to be almost an ideal selectable marker gene. The characteristics of such an ideal marker are given in Table 8 and are compared with the characteristics associated with NPT II. Herbicide resistance genes have also been used as selectable markers. They are discussed in section 4.4.

To express these marker genes in plants, the coding regions have been fused to plant promoter, enhancer, and termination sequences. A diagram of a plant gene is presented in Figure 8. These sequences are often removed from the genes of the

Ti plasmid for nopaline synthase (Bevan et al. 1983, Depicker et al. 1983), octopine synthase (De Greve et al. 1983), and mannopine synthase (Di Rita and Gelvin 1987), as well as those from the 19S and 35S gene transcripts of the cauliflower mosaic virus (Odell et al. 1985, Lawton et al. 1987). The promoter and enhancer sequences of these genes were thought to give constitutive expression of the chimeric genes in transgenic plant tissues (Kozziel et al. 1984, Shaw et al. 1984, Odell et al. 1985, An 1986, Di Rita and Gelvin 1987). However, tissue or cell specificity was reported for the 35S promoter (Nagata et al. 1987). The 35S promoter promotes a higher level of transcription in transgenic *N. tabacum* than the nopaline synthase promoter (Sanders et al. 1987, Harpster et al. 1988).

Table 8. Characteristics of an ideal selectable marker for plant transformation compared with the characteristics of the neomycin phosphotransferase II (NPT II) gene (in parentheses)

- | | |
|----|---|
| 1. | The selective agent (antibiotic) must provide stringent selection.
(NPT II is relatively stringent when used with kanamycin; however, there are reports of leakiness.) |
| 2. | The selective agent should not interfere with regeneration of transgenic plants.
(Kanamycin does not interfere, but slows down regeneration in several instances.) |
| 3. | The selectable marker should permit the use of different selective agents.
(NPT II allows for the use of kanamycin, neomycin, paromomycin, and geneticin.) |
| 4. | The selection process should work with a wide range of plant species.
(NPT II has been the most widely used selectable marker; see Table 5.) |
| 5. | The selectable marker gene should be available in different gene constructions.
(NPT II has been constructed with a large number of different vectors and regulatory sequences.) |
| 6. | The activity of the selectable marker gene product must be detectable.
(NPT II can be detected using a gel assay or a dot blot assay.) |
| 7. | The selective agent should be inexpensive and readily available.
(Kanamycin, neomycin, and geneticin are all inexpensive and available through regular chemical suppliers.) |

3.5 Confirmation of stable genetic transformation

An important step in the regeneration of transgenic plants is the confirmation of stable gene integration in the plant genome. Ideally, this should be confirmed through phenotypic and physical evidence of the presence of the introduced genes in the original transgenic plant and its progeny. Proper control should be maintained to prevent interference by contaminants or endogenous microorganisms. The introduced genes, in a Southern analysis,

should be present with the high molecular weight DNA and, when appropriate, rearrangement of the integrated sequences should be shown. Gene activity should be detectable either enzymatically or phenotypically. In Table 5, some of the references listed involving transgenic species (in particular the monocots) do not possess all of the elements required to confirm stable transformation. For a critical review, consult Potrykus (1990) who assessed gene transfer in cereals.

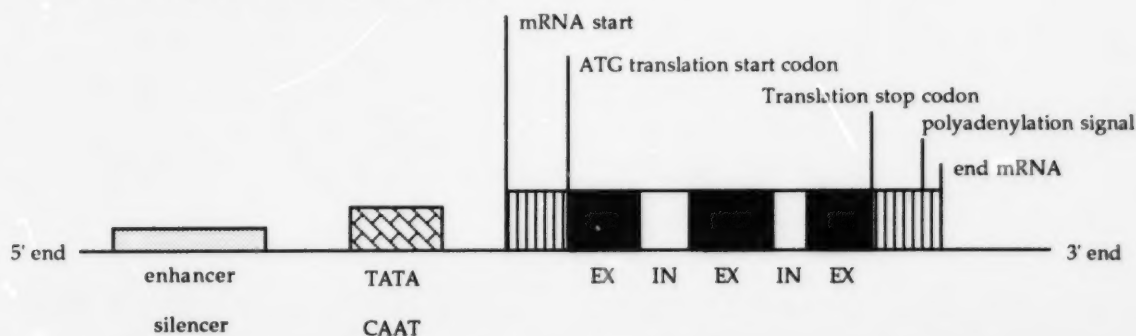


Figure 8. Schematic of a plant gene. A typical plant gene consists of three regions: the promoter-regulator region, which comprises the enhancer or silencer elements that are responsible for regulating gene expression, and the TATA and CAAT boxes, which initiate gene processing. The coding region consists of the mRNA start site, the ATG translation start codon, the exons (which are DNA sequences that will be translated into protein), and the introns (which are DNA sequences that will be processed out of the mRNA). The 3' end terminal region ensures proper termination of the gene sequence when translated and transcribed. The poly A addition site is where poly A polymerase adds a poly A tail to mRNA.

These criteria must be accounted for to confirm stable genetic transformation; however, in certain plant systems, not all of them can easily be obtained (see section 5.3).

3.6 Physiological effects of foreign gene introduction

To date, no significant effects of marker genes (screenable or selectable) have been reported on the overall physiology of transgenic plants. Although, morphological changes in regenerants have been observed it is not clear if they are due to the regeneration process or to integration of the gene itself.

4. GENETIC ENGINEERING OF PLANTS WITH GENES OF AGRONOMIC VALUE

Genetic engineering of plants with genes of agronomic value has been limited to single gene traits. Resistance to virus disease, insect species, herbicide, and heavy metal have been engineered in model plant systems such as *N. tabacum*, *P. hybrida*, *L. esculentum*, and *A. thaliana*. Engineering of fungal disease resistance has been attempted, with no conclusive results. Genetic manipulation for bacterial disease resistance and for growth regulation is now possible. This part of our literature review will deal with accomplishments using agronomic traits for plant genetic engineering.

4.1 Viral disease resistance

Viral disease resistance in transgenic plants has been obtained through three different mechanisms (Table 9). The first mimics the phenomenon of virus cross protection, which is the infection of plant with an avirulent or a mild strain of a virus that prevents the development of severe symptoms when the same plant is infected with a severe strain of the same virus (Fulton 1986). This has been achieved in transgenic plants by cloning the virus coat protein gene cDNA and integrating it into the plant genome. A summary of transgenic plants with engineered virus disease resistance is given in Table 9 and an exhaustive review by Beachy et al. (1990) should be consulted on coat protein-mediated resistance. In all cases using this approach, a delay in systemic spreading of the virus and a decrease in the number of lesions and

plants with symptoms have been observed in transgenic plants. The induced resistance could be due to the blockage of initial virion uptake into the cell or to the prevention of uncoating once the virion is inside the cell (Tumer et al. 1987, Cuozzo et al. 1988, Hemenway et al. 1988). It has also been postulated that the coat protein may inhibit viral RNA transcription (Loesch-Fries et al. 1987) and viral spreading from cell to cell (Cuozzo et al. 1988). This kind of protection can be partially overcome by using an RNA inoculum instead of virions (Tumer et al. 1987). In the case of potato virus X (PVX) protection, the RNA inoculum did not overcome the protection as seen with the other virus (Hemenway et al. 1988). The PVX coat protein or its RNA are thought to inhibit early events during RNA infection. Field tests with transgenic *L. esculentum* plants expressing a tobacco mosaic virus (TMV) coat protein gene have shown that resistance to TMV and ToMV (tomato mosaic virus) can be achieved (Nelson et al. 1988).

The second mechanism of protection used in transgenic plants is the introduction of an antisense gene to the virus coat protein gene. The antisense RNA could hybridize either to the coat protein of the infecting virus or to its RNA and prevent replication. This approach has been used in *N. tabacum* for cucumber mosaic virus (CMV) (Cuozzo et al. 1988), with PVX (Hemenway et al. 1988), and with tobacco rattle virus (TRV) (Powell et al. 1989). However, the resistance obtained is functional at a low level of inoculum only.

The third strategy is based on the introduction of a gene coding for virus satellite RNA present in plant RNA viruses, which suppresses the symptoms of virus infection. Cloned satellite RNA expressing their DNA with CMV (Harrison et al. 1987) and with the tobacco ring spot virus (TobrV) (Gerlach et al. 1987) in *N. tabacum* showed a decrease in the intensity of symptoms. Interaction of the satellite RNA with the viral RNA is a possible explanation for this type of tolerance. This approach has major limitations because satellite RNAs are found only in some RNA viruses; thus, resistance is host specific and virus specific.

4.2 Insect resistance

Insect resistance in transgenic plants has been achieved using two different types of genes. In both instances, *A. tumefaciens*-mediated

Table 9. Virus protection in transgenic plants

Mechanism	Virus	Plant	Reference
Satellite RNA	Cucumber mosaic virus (CMV)	<i>N. tabacum</i>	Baulcombe et al. (1986), Harrison et al. (1987)
Coat protein		<i>N. tabacum</i>	Cuozzo et al. (1988)
Antisense to CP		<i>N. tabacum</i>	
Coat protein	Tobacco mosaic virus (TMV)	<i>L. esculentum</i>	Nelson et al. (1988)
Coat protein	Tomato mosaic virus (ToMV)	<i>N. tabacum</i>	Powell-Abell et al. (1986)
Coat protein	Alfalfa mosaic virus	<i>N. tabacum</i>	Loesch-Fries et al. (1987)
Coat protein	(AIMV)	<i>N. tabacum</i>	Tumer et al. (1987)
		<i>L. esculentum</i>	
Coat protein	Potato virus X (PVX)	<i>N. tabacum</i>	Hemenway et al. (1988)
Antisense			
Satellite RNA	Tobacco ring spot virus (TobrV)	<i>N. tabacum</i>	Gerlach et al. (1987)
Coat protein of soybean mosaic virus	Tobacco etch virus (TEV)	<i>N. tabacum</i>	Stark and Beachy (1989)
Coat protein	Tobacco streak virus (TSV)	<i>N. tabacum</i>	Van Dun and Bol (1988)
Coat protein	Tobacco rattle virus (TRV)	<i>N. tabacum</i>	Van Dun et al. (1988)
Antisense to CP		<i>N. tabacum</i>	Powell et al. (1989)
Coat protein	Potato viruses X	<i>S. tuberosum</i>	Lawson et al. (1990)

transformation was used to obtain transgenic plants. The first type comprises genes encoding insect toxins produced by *Bacillus thuringiensis*. During bacterial sporulation, these toxins are produced as crystalline proteinaceous inclusion bodies. They are dissolved by alkaline conditions in the insect midgut (Aronson et al. 1986) and cleaved to yield peptides only toxic to insects. Different strains of *B. thuringiensis* exist and they are active against specific insects. The strain *israelensis* acts against *Diptera*, strains *kurstaki* and *berliner* act against *Lepidoptera* and strains *tenebrionis* and *san diego* act against *Coleoptera* (Aronson et al. 1986).

A truncated toxin gene of *B. thuringiensis* var. *berliner* was used to produce transgenic *N. tabacum* resistant to the insect larvae of *Manduca sexta* (tobacco hornworm) (Vaecck et al. 1987). Use of a truncated gene was necessary to obtain biologically significant levels of toxin proteins. The level of resistance was dependent upon the level of gene expression. Similar results were obtained with the *B. thuringiensis* var. *kurstaki* toxin gene in transgenic *N. tabacum* (Barton et al.

1987), which was resistant to the tobacco hornworm. Furthermore, insect mortality increased and plant damage by other insects decreased (cotton bollworm, *Heliothis virescens*; corn earworm, *Heliothis zea*; beet armyworm, *Spodoptera exigua*). A truncated form of the same gene used in transgenic *L. esculentum* reduced the damage by the larvae of the tobacco hornworm (Fischhoff et al. 1987, Delannay et al. 1989). Transgenic *L. esculentum* plants were also resistant to *H. virescens* and *H. zea*.

The other class of engineered genes encodes the proteinase inhibitors present in the leaves of various plants. These genes encode naturally occurring proteins induced by insect attack. They inhibit the action of digestive enzymes in insects and also in animals. They are nonspecific in terms of the insects or animals they affect, but they are specific for certain classes of digestive enzymes (Table 10; for a review of these inhibitors see Garcia-Olmedo et al. (1987)). Proteinase inhibitor genes have been isolated from tomato, potato, and cowpea (Hilder et al. 1987, Ryan and An 1988). Transgenic *N. tabacum* plants that express a

Table 10. Protein inhibitors of proteinases

Inhibitor group	Enzymes inhibited
Soybean trypsin inhibitors	Serine proteinases and endogenous α -amylases
Bowman Birk inhibitors	Serine proteinases
Cereal trypsin inhibitors	Serine proteinases and heterologous α -amylases
Potato inhibitors I and II	Serine proteinases
Squash inhibitors	Serine proteinases
Cathepsin D and pepsin inhibitors	
Cowpea trypsin inhibitors	Serine inhibitors

cowpea trypsin inhibitor gene have been obtained via *A. tumefaciens* transformation (Hilder et al. 1987). The transgenic plants were tolerant to tobacco budworm (*Heliothis virescens*). The advantage of this approach is the potential for resistance to a wide range of insects (*Heliothis*, *Spodoptera*, *Diabrotica*, and *Tribolium*) in contrast to the *Bacillus thuringiensis* toxin genes, which provides narrower resistance.

4.3 Heavy metal resistance

Some soils have been contaminated with heavy metals such as Cd, Hg, Zn, and Cu through industrial and agricultural pollution (Davis 1984, Poon 1984). Vegetables and forage crops that sequester heavy metals in specific tissues not consumed by humans or animals may offer us protection. Plants have their own Cd-binding proteins (phytochelatin) (Grill et al. 1985) and, by genetic engineering, the amount of metal-binding proteins in transgenic plants can be increased.

A chimeric mouse metallothionein gene which binds heavy metals has been transferred by *A. tumefaciens*-mediated transformation to transgenic *N. tabacum* (Maiti et al. 1988); however, the transgenic gene has not been tested for metal binding. In a different study, the Chinese hamster metallothionein II gene was inserted into an engineered cauliflower mosaic virus (CaMV) vector and *B. campestris* plants were infected (Lefebvre et al. 1987). The virus-infected plants showed Cd tolerance and depleted Cd pools in leaf tissues. Increased Cd tolerance was obtained in transgenic *B. napus* and *N. tabacum* plants engineered with a chimeric human metallothionein gene (MT-II; Misra and Gedamu 1989). Results indicate the feasibility of achieving heavy metal tolerance through genetic transformation in plants. Together, these studies

show that an animal metallothionein may function in transgenic plants.

4.4 Herbicide resistance

Herbicide resistance in plants has particular commercial importance. Herbicide resistance in transgenic plants can be achieved by three different strategies: the introduction of a mutant gene coding for an enzyme with a modified herbicide binding site, the introduction of a chimeric gene that overproduces the herbicide target enzyme, and the introduction of a gene coding for an enzyme that detoxifies the herbicide (Table 11). All three approaches have been exploited.

The strategy involving use of herbicide-resistant mutant genes has been used successfully with the herbicides glyphosate (Monsanto) and chlorsulfuron (DuPont). Glyphosate (or N-phosphonomethyl glycine) is a broad spectrum herbicide acting against the 5-enolpyruvylshikimate-3-phosphate synthase enzyme (EPSP synthase) involved in the synthesis of aromatic amino acids (Steinrucken and Amrhein 1980). When the herbicide is active, shikimate is not processed and accumulates, resulting in starvation of the aromatic amino acids. A mutant gene from the bacteria *Salmonella typhimurium* has been isolated in which substitution of a proline for a serine in the enzyme resulted in decreased affinity of the herbicide for the enzyme (Comai et al. 1985). This gene was fused to octopine synthase and mannopine synthase promoter sequences, and then introduced into *N. tabacum* and *L. esculentum* to give tolerance to the herbicide glyphosate. The transgenic plants were resistant to the herbicide, but yield was reduced by 30 to 70% (Comai et al. 1985, Fillatti et al. 1987b). Using the same approach similar results were obtained in transformed *B. campestris* tumour tissues (Thompson et al. 1987b).

A gene encoding a chlorsulfuron-resistant acetolactate synthase (*csr1-1*) isolated from the mutant *A. thaliana* line GH50 has been used to recover transgenic *N. tabacum* plants (Haughn et al. 1988, Gabard et al. 1989, Charest et al. 1990). This gene also conferred resistance to sulfonyleureas. The two herbicides are members of the sulfonyleureas, a new class of herbicides inhibiting acetolactate synthase (ALS), an essential enzyme in the pathway for branched-chain amino

Table 11. Genetic engineering of herbicide resistance in plants

Herbicide	Plant	Mechanism	Gene	Reference
Chlorsulfuron	<i>N. tabacum</i>	Mutation	acetolactate synthase	Haughn et al. (1988), Lee et al. (1988), Gabard et al. (1989) Charest et al. (1990)
	<i>L. esculentum</i>	Mutation	acetolactate synthase	Mazur et al. (1987a,b)
	<i>M. sativa</i>			D'Halluin et al. (1990)
	<i>B. napus</i>			Miki et al. (1990)
Glyphosate	<i>N. tabacum</i>	Mutation	aroA	Comai et al. (1985)
	<i>B. campestris</i> *	Mutation	aroA	Thompson et al. (1987b)
	<i>L. esculentum</i>	Mutation	aroA	Fillatti et al. (1987a)
	<i>P. hybrida</i>	Overexpression	EPSP synthase	Shah et al. (1986)
	<i>A. thaliana</i>	Overexpression	EPSP synthase	Klee et al. (1987b)
Phosphinotricin	<i>L. esculentum</i>	Detoxification	Phosphinotricin, acetyltransferase	De Block et al. (1987)
		Overexpression	glutamine synthetase	Eckes et al. (1989)
Bromoxynil	<i>N. tabacum</i>	Detoxification	Nitrilase	Stalker et al. (1988)
2,4-D	<i>N. tabacum</i>	Detoxification	2,4-D monooxygenase	Streber and Willmitzer (1989)
	<i>N. tabacum</i>	Detoxification	2,4-D monooxygenase	Lyon et al. (1989)
Atrazine	<i>N. tabacum</i>	Mutation	psbA	Cheung et al. (1988)

* *B. campestris* tumour tissues.

acid biosynthesis (Ray 1986). The ALS enzyme, although coded by a nuclear gene, is localized in the chloroplast, the site of amino acid biosynthesis (Mazur et al. 1987a,b). Similar results were obtained with two *N. tabacum* mutant genes (C3 of the *SuRA* locus and Hra-S4 of the *SuRB* locus). Transgenic *N. tabacum* and *L. esculentum* were resistant to high levels of chlorsulfuron and sulfometuron methyl (Mazur et al. 1987a,b, Lee et al. 1988).

The second approach to herbicide resistance in transgenic plants involves overexpression of the target enzyme. This approach has been used to achieve resistance to the herbicide glyphosate. The EPSP synthase gene isolated from an overproducer cell line of *P. hybrida* was fused to the CaMV 35S promoter and reintroduced into *P. hybrida*. The transgenic plants were resistant to more than four times the glyphosate dose required to kill plants (Shah et al. 1986). A similar strategy was used with an EPSP synthase gene from *A. thaliana*, isolated with the *P. hybrida* EPSP synthase gene as the probe. The *A. thaliana* EPSP synthase gene was fused to 35S promoter sequences and reintroduced into *A. thaliana*. The transgenic plants were resistant to glyphosate *in vitro* (Klee et al. 1987b).

In plants, the EPSP gene is a nuclear-encoded gene synthesized as a cytoplasmic precursor and

targeted at the chloroplast (Della-Cioppa et al. 1987). This could explain why Comai et al. (1985) only observed tolerance and not complete resistance to glyphosate with the bacterial *AroA* gene. Tolerance is defined here as survival of the plants with a delay in growth, whereas resistance is defined as the complete absence of symptoms of toxicity. The use of plant genes with native transit peptide sequences (Shah et al. 1986, Klee et al. 1987) may have ensured transport to the chloroplast, whereas the *AroA* encoded enzyme lacked a transit sequence and may have been restricted to the cytoplasm. Overexpression of the target enzyme has also been used to confer resistance to L-phosphino-tricin in transgenic *N. tabacum* plants (Eckes et al. 1989). An alfalfa (*Medicago sativa*) glutamine synthetase gene was linked to the 35S promoter of CaMV to achieve overexpression of this enzyme and herbicide tolerance (Eckes et al. 1989). The transgenic plants were resistant to about four times field application levels.

The third strategy is to use a detoxifying enzyme gene in transgenic plants to achieve herbicide resistance. This approach was used in transgenic *N. tabacum*, *L. esculentum*, and *S. tuberosum* plants to obtain resistance to phosphinotricin and bialaphos (De Block et al. 1987). Bialaphos is an antibiotic produced by

Streptomyces hygroscopicus (Imai et al. 1985), and is nearly identical to phosphinotricin, except that it has two alanine residues. The phosphinotricin is chemically synthesized and is an analogue of glutamic acid (Bayer et al. 1972). These two herbicides are inhibitors of the glutamine synthetase enzyme, which plays a major role in the assimilation of ammonia and the regulation of nitrogen metabolism in plants (Tachibana et al. 1986). When the enzyme is inhibited by one of these two herbicides, ammonia is accumulated and kills the plant cells. The detoxification gene was isolated from *S. hygroscopicus* (Thompson et al. 1987a). This gene (*bar*) encodes a phosphinotricin acetyltransferase enzyme, which acetylates the free NH₂ group of phosphinotricin and bialaphos and prevents their action. The *bar* gene was fused to 35S promoter sequences and introduced into *N. tabacum*, *L. esculentum*, and *S. tuberosum* to give resistance to phosphinotricin (Basta) and bialaphos (Herbiacea) up to a dose of 20 L/ha, which is three to eight times the level used in the field (De Block et al. 1987).

Similar work has been carried out using a detoxification mechanism in transgenic *N. tabacum* to give resistance to bromoxynil (3,5-dibromo-4-hydroxybenzonitrile; Stalker et al. (1988)) and to 2,4-D (4-chloro-2-methyl phenoxyacetic acid; Lyon et al. (1989), Streber and Willmitzer (1989)). In the case of resistance to bromoxynil, a gene (*bxn*) encoding a nitrilase, which converts bromoxynil into inactive chemicals, was isolated from the soil bacterium *Klebsiella ozaenae* and hooked up to a light-regulated promoter (RuBP) for genetic transformation in *N. tabacum*. The progeny of the transgenic plants was resistant to field-level applications of the herbicide (0.57 kg/ha). Finally, with the herbicide 2,4-D, resistance was achieved in transgenic plants by using a detoxification gene (*tfdA*; 2,4-dichlorophenoxyacetate monooxygenase (DPAM); Lyon et al. (1989), Streber and Willmitzer (1989)) from the soil bacterium *Alcaligenes eutrophus* linked to a plant promoter (35S). The *N. tabacum* transgenic plants were resistant to field-level applications of 2,4-D (1-2 kg/ha).

4.5 Fungal disease resistance

Fungus resistance has been attempted using chitinase genes isolated from *Serratia marcescens* (Jones et al. 1986) in *A. tumefaciens*-mediated transformation experiments. Resistance was

expected because chitinase hydrolyzes chitin in such plant pathogens as *Fusarium oxysporum* (Jones et al. 1986). Transgenic plants were obtained with *P. hybrida* (Jones et al. 1985) and *N. tabacum* (Jones et al. 1988), but an assessment of fungus resistance was not made. A similar attempt was made by using a chitinase gene from *N. tabacum* under the control of the 35S promoter that was introduced in *N. sylvestris* (Neuhaus et al. 1991). The transgenic plants were challenged by the fungal pathogen *Cercospora nicotiana* and no increased resistance was observed.

4.6 Bacterial disease resistance

Insect antibacterial protein genes exhibit a potential for use in the genetic engineering of plant species. Insects possess a humoral immunity system that is induced by bacterial infection. Some families of antibacterial peptides have been characterized to the gene level, such as the lysozymes, cecropins, and attacins of the giant silk moth (*Hyalophora cecropia*) and the apidaecins of honey bees (*Apis mellifera*) (Jaynes et al. 1987, Casteels et al. 1989). They will be used in the near future to engineer crop plants to render them resistant to bacterial disease.

Another approach used successfully in *N. tabacum* involved introducing a bacterial gene (*ttr* for tabtoxin resistance) encoding an enzyme that acetylates tabtoxin of *Pseudomonas syringae* pv. *tabaci*. This toxin contributes to wildfire disease and induces chlorotic symptoms. Expression of the *ttr* gene in transgenic plants gave resistance to the tabtoxin (Anzai et al. 1989, 1990, Anzai and Yoneyama 1990), a demonstration of the feasibility of engineering bacterial disease resistance in plants.

4.7 Growth regulators

Growth regulator genes have been isolated from the Ti and Ri plasmids of *Agrobacterium*. They encode genes involved in the biosynthesis of auxins and cytokinins (section 3.3.1.a). The isopentenyl transferase gene of the Ti plasmid of *A. tumefaciens*, encoding an enzyme in the cytokinin biosynthetic pathway, has been used in combination with a heat shock promoter to modify the development of transgenic *N. tabacum* and *A. thaliana* plants (Medford et al. 1989). The resulting plants had reduced height, leaf area, and stem width.

Two genes coding for enzymes producing the auxin indole acetic acid were introduced into *P. hybrida* via *A. tumefaciens*-mediated transformation (Klee et al. 1987a). Transgenic plants in which genes were under the control of a constitutive promoter had abnormal morphology. The plants had smaller, curled leaves and adventitious roots. There was no branching, which is evidence of strong apical dominance, and stems were elongated and woody. This demonstrates that genetic engineering can be used to modify the overall shape and appearance of a plant. Similar phenomena have been observed with wild type genetic transformation using the Ri plasmid where transgenic plants had short internodes, wrinkled leaves, plagiotropic roots, and reduced fertility (Guerche et al. 1987c). The use of engineered Ti plasmid avoids the extreme problems of reduced fertility and plagiotropic roots.

Antisense technology has been used successfully to inhibit synthesis of the hormone ethylene in transgenic *L. esculentum* plants (Hamilton et al. 1990). This was done by introducing a chimeric antisense gene to an unknown gene encoding a protein found to be correlated with ethylene production in *L. esculentum*. Expression of the antisense gene reduced the level of ethylene production. It was postulated that the unknown gene was involved in the conversion of 1-aminocyclopropane-1-carboxylic acid to ethylene. This suggests the possibility of regulating the production of this hormone which is involved in abscission, senescence, and fruit ripening.

4.8 Fruit and vegetable quality

Transgenic *L. esculentum* plants were produced using an antisense polygalacturonase gene (Smith et al. 1988, Kramer et al. 1989) that reduced the expression of the endogenous gene in tomato fruits. This gene product is important for fruit softening caused by solubilization of the pectin fraction of the cell wall. In a complementary study (Smith et al. 1990), inhibition of pectin depolymerization, lycopene accumulation, polyuronide solubilization, invertase activity, and pectinesterase activity were demonstrated, but there was no effect on ethylene production.

Genetic engineering may also be used to improve the nutritional quality of food plants. In *Solanum tuberosum*, a 292 basepair synthetic

chimeric gene, coding for an essential amino acid, fused to a NOS promoter - CAT gene construction was introduced by *Agrobacterium*-mediated transformation and was expressed in all parts of the transgenic potato plants (Yang et al. 1989). It was found that 0.02 to 0.35% of the total protein was fusion protein. However, no data on improved protein quality were given. In a similar experiment, a methionine-rich seed protein from a Brazil nut (*Bertholletia excelsa*) gene was transferred to *N. tabacum* and was expressed in the seed (Altenbach et al. 1989). Similar results were obtained with transgenic *B. napus* (Guerche et al. 1990). A 30% increase in methionine content was measured in transgenic *N. tabacum* seeds. This suggests the possibility of improving or altering the nutritional quality of seed proteins.

The sweetness of vegetables and fruits could also be modified. The gene encoding for the sweet protein thaumatococin has been used in transformation experiments to produce transgenic *S. tuberosum*. The gene was expressed in leaves and was detected by the intense sweet taste of the transgenic tissues (Witty 1989). The two probable applications are 1) in improvement of sweetness in food and 2) derivation of a new screenable marker for transformed tissues.

4.9 Flower colour modification

Flower colour modification has been accomplished in *Petunia hybrida* by transferring the dihydroquercetin 4-reductase gene of *Zea mays* into *P. hybrida* mutant plants that had no pigmentation (Meyer et al. 1987, Mol et al. 1989). The result was a novel red pigmentation, due to the constitutive expression with a 35S promoter from CaMV of the gene in transgenic plants that produces an enzyme in the flavonoid pathway. Another approach taken was the use of an antisense RNA gene against a chalcone synthase gene in *P. hybrida* transgenic plants. Inhibition of flower pigmentation at various levels was observed (Van derKrol et al. 1990).

4.10 Production of bioactive chemicals

Production of a biologically active peptide has been genetically engineered in *B. napus* and *A. thaliana* using *Agrobacterium tumefaciens*-mediated transformation (Venderkerckhove et al. 1989). This was accomplished by using the seed specific gene encoding 2S albumin in *A. thaliana* and introducing into its coding region the pentapeptide gene encoding enkephalin (a

neuropeptide with opiate activity), with tryptic cleavage sites at each end of the neuropeptide. The chimeric gene was expressed specifically in the seed and the peptide was isolated through protein extraction and enzymatic cleavage. The transgenic plants of both species produced up to 200 nmol of peptide per gram of seeds.

Production of functional antibodies was achieved in transgenic tobacco through the transfer of chimeric genes encoding gamma and kappa immunoglobulin chains of mouse hybridoma. The antibodies were produced up to level of 1.3% of the total leaf protein (Hiatt et al. 1989). This opens the door to producing antibodies in plants to bind specific molecules, such as herbicides or environmental contaminants.

4.11 Flower sterility

With the characterization of tissue- or stage-specific genes, new avenues have opened for plant genetic engineering. Obtaining male sterility in transgenic plants is an excellent example of a basic application (Peacock 1990). A tissue-specific gene expressed only in anther tapeta was characterized and its promoter used in combination with two RNase genes to produce chimeric "suicide" genes that kill tapetal cells when expressed (Mariani et al. 1990). Male sterility was obtained in both transgenic *N. tabacum* and *B. napus* plants using this approach. This type of technology will be extremely useful in applied breeding programs.

5. APPLICATION OF GENETIC ENGINEERING TO TREE SPECIES

5.1 Tree genetic transformation

Accomplishments in tree genetic transformation can be separated into two groups. The first group comprises the angiosperm species, in particular all dicots. In this group, it can be assumed that the basic principles of gene regulation and gene expression are the same as in crop plants. The second group, includes the gymnosperms (conifers only). They are more primitive and there are still many questions about their gene regulation and gene expression. Earlier studies (Smith 1935a,b, 1936, 1937, 1938, 1939, 1942, De Cleene and De Ley 1976) have revealed crown gall infection on tree species but there was no realization that these tumors were caused by genetic transformation.

Transgenic plants have been obtained from the following angiosperms: *Juglans regia* (walnut), *Malus pumila* (apple), *Populus alba* x *grandidentata* (poplar), *P. trichocarpa* x *deltoides*, and *P. alba* x *tremula* (aspen) using *A. tumefaciens* in all cases (Table 12). Indications of transgenic plant recovery have been reported for *Azadirachta indica* (neem) and *Citrus jambhiri*, but no final proof has been provided (see section 3.5). *A. tumefaciens* has been used to produce transformed tissues (tumours) on a number of other species (Table 12). Electroporation has been used with *Alnus incana*, but only transient expression was achieved.

With the gymnosperms no transgenic plants have been recovered (Table 13). *A. rhizogenes* and *A. tumefaciens* have been used to produce tumours on seedlings of various species and electroporation has been used to obtain transient expression in protoplasts of *Picea glauca* (white spruce), *Picea mariana* (black spruce), *Pinus banksiana* (jack pine), *Pinus taeda* (loblolly pine), *Pseudotsuga menziesii* (Douglas-fir), and *Larix eurolepis* (larch), but no stable transformation was achieved (Table 13). Interestingly, opines have been detected in tumour tissues, which indicated that these genes, with their promoters, are functional in gymnosperm tree species (Sederoff et al. 1986, Dendekar et al. 1987, Diner and Karnosky 1987, Ellis et al. 1989, Loopstra et al. 1990). Similarly, the 35S promoter of CaMV hooked up to the *Lux* gene was expressed in *Pseudotsuga menziesii* tumours (Ellis et al. 1989). These results demonstrate the possibility of using chimeric genes designed for crop plants in gymnosperm tree genetic transformation.

Research dealing with the recovery of tree transgenic plants is recent, as indicated by the references listed in Tables 12 and 13 (mostly 1988 and 1989 papers). Although the recovery of transgenic plants has not been possible with gymnosperm tree species, the development of somatic embryogenesis and protoplast systems in conifers will allow the recovery of transgenic plants. Genetic transformation is possible via *Agrobacterium*, which is an important factor for the success of transgenic plant recovery. Other methods should also be investigated to allow the use of different types of tissues with alternative transformation protocols (i.e., microinjection and microprojection).

Table 12. Genetic transformation of Angiosperm* tree species.

Species	Common name	Method	Results	Reference
<i>Allocauarina verticillata</i>		<i>A. rhizogenes</i>	Transgenic trees (Hairy root regenerants)	Phelep et al. (1991)
<i>Alnus glutinosa</i>	Alder	<i>A. tumefaciens</i>	Tumours on shoots <i>in vitro</i>	Mackay et al. (1988)
<i>Alnus incana</i>	Alder	<i>A. tumefaciens</i>	Tumours on shoots <i>in vitro</i>	Mackay et al. (1988)
<i>Alnus incana</i>	Alder	Electroporation	Transient expression (GUS)	Séguin and Lalonde (1988)
<i>Azadirachta indica</i>	Neem	<i>A. tumefaciens</i>	Shoots resistant to km	Naina et al. (1989)
<i>Betula papyrifera</i>	Birch	<i>A. tumefaciens</i>	Tumours on shoots <i>in vitro</i>	Mackay et al. (1988)
<i>Carica papaya</i>	Papaya	<i>A. tumefaciens</i>	Transgenic tissues resistant to km	Pang and Sanford (1988)
<i>Citrus jambhiri</i>	Rough lemon	PEG delivery	Transgenic plants resistant to km	Vardi et al. (1990)
<i>Citrus sinensis</i>	Orange	PEG delivery	Calli resistant to km	Kobayashi and Uchimiya (1989)
<i>Citrus sinensis</i>	Orange	<i>A. tumefaciens</i>	Transgenic calli and putative transgenic plantlet resistant to km and Hyg	Hidaka et al. (1990)
<i>Juglans regia</i>	Walnut	<i>A. tumefaciens</i>	Transgenic plants resistant to km	McGranahan et al. (1988)
<i>Malus pumila</i>	Apple	<i>A. tumefaciens</i>	Transient expression (GUS)	Martin et al. (1990)
<i>Malus pumila</i>	Apple	<i>A. tumefaciens</i>	Transgenic plants resistant to km	James et al. (1989)
<i>Populus trichocarpa</i> x <i>deltoidea</i>	Poplar	<i>A. tumefaciens</i>	Tumours <i>in vitro</i>	Parsons et al. (1986)
		<i>A. rhizogenes</i>	Roots and plantlets resistant to km	Phytoud et al. (1987)
<i>Populus alba</i> x <i>grandidentata</i>	Poplar	<i>A. tumefaciens</i>	Transgenic plants resistant to km	Fillatti et al. (1987b)
			Tumours <i>in vitro</i> resistant to km	Chun et al. (1988)
<i>Populus alba</i> x <i>tremula</i>	Aspen	<i>A. tumefaciens</i>	Transgenic plants resistant to km and PPT	De Block (1990)
<i>Populus trichocarpa</i> x <i>deltoidea</i>	Poplar	<i>A. tumefaciens</i>	Transgenic plants resistant to km and PPT	De Block (1990)
<i>Prunus persica</i>	Peach	<i>A. tumefaciens</i>	Tumours <i>in vitro</i>	Hammerschlag et al. (1989)
<i>Prunus persica</i>	Peach	<i>A. tumefaciens</i>	Transient expression (GUS)	Martin et al. (1990)
		<i>A. rhizogenes</i>		
<i>Prunus persica</i>	Peach	<i>A. tumefaciens</i>	Calli resistant to km, G418	Scorza et al. (1990)
<i>Robinia pseudoacacia</i>	Black locust	<i>A. tumefaciens</i>	Tumours on hypocotyl segments <i>in vitro</i>	Davis and Keathley (1989)
		<i>A. rhizogenes</i>	Tumours resistant to km	
<i>Salix schaverinii</i>	Willow	<i>A. tumefaciens</i>	Tumours <i>in vitro</i> and in plants	Vahala et al. (1989)
<i>Salix viminalis</i>				
<i>Salix dasyclados</i>				
<i>Vitis rupestris</i>	Grapevines	<i>A. tumefaciens</i>	Trangenic (GUS+NPT II) plants	Mullins et al. (1990)
<i>Vitis vinifera</i>			Transformed buds (GUS+NPT II)	

* All of the species listed are dicots. Abbreviations km = kanamycin, Hyg = hygromycin, GUS = β -glucuronidase, NPT II = neomycin phosphotransferase, PPT = phosphinotricin.

Table 13. Genetic transformation of gymnosperm* tree species

Species	Common name	Method	Results	Reference
<i>Abies nordmanniana</i>	Caucasian fir	<i>A. tumefaciens</i>	Tumours on seedlings	Clapham and Ekberg (1986)
<i>Abies procera</i>	Nobel fir	<i>A. tumefaciens</i> <i>A. rhizogenes</i>	Tumours on seedlings	Morris et al. (1989)
<i>Larix decidua</i>	European larch	<i>A. rhizogenes</i>	Roots on <i>in vitro</i> grown stem	Diner and Karnosky (1987), Karnosky et al. (1988)
<i>Larix eurolepis</i>	Hybrid larch	Electroporation	Transient expression of GUS and CAT	Charest et al. (1991)
<i>Libocedrus decurrens</i>	Incense cedar	<i>A. tumefaciens</i>	Tumours on plants	Stomp et al. (1990)
<i>Picea abies</i>	Norway spruce	<i>A. tumefaciens</i>	Tumours on seedlings	Clapham and Ekberg (1986), Clapham et al. (1990), Hood et al. (1990)
<i>Picea glauca</i>	White spruce	Electroporation	Transient expression of CAT and GUS	Wilson et al. (1989), Bekkaoui et al. (1988)
		<i>A. tumefaciens</i>	Tumours on seedlings	Ellis et al. (1989)
<i>Picea engelmanni</i>	Engelmann spruce	<i>A. tumefaciens</i>	Tumours on seedlings	Ellis et al. (1989)
<i>Picea mariana</i>	Black spruce	Electroporation	Transient expression of CAT	Tautorus et al. (1989)
<i>Picea mariana</i>	Black spruce	Microprojection	Transient expression of GUS	Duchesne and Charest (1991)
<i>Picea sitchensis</i>	Sitka spruce	<i>A. tumefaciens</i>	Tumours on seedlings	Ellis et al. (1989)
<i>Pinus banksiana</i>	Jack pine	Electroporation	Transient expression of CAT	Tautorus et al. (1989)
<i>Pinus eldarica</i>	Afghan pine	<i>A. tumefaciens</i>	Tumours on plants	Stomp et al. (1990)
<i>Pinus elliotti</i>	Slash pine	<i>A. tumefaciens</i>	Tumours on plants	Stomp et al. (1990)
<i>Pinus jeffreyi</i>	Jeffrey pine	<i>A. tumefaciens</i>	Tumours on plants	Stomp et al. (1990)
<i>Pinus lambertiana</i>	Sugar pine	<i>A. tumefaciens</i>	Tumours on plants Calli resistant to km and expressing NPT II	Stomp et al. (1990) Loopstra et al. (1990)
<i>Pinus radiata</i>	Monterey pine	<i>A. tumefaciens</i>	Tumours on plants	Stomp et al. (1990)
<i>Pinus sylvestris</i>	Scots pine	<i>A. tumefaciens</i>	Tumours on plants	Stomp et al. (1990)
<i>Pinus virginiana</i>	Virginia pine	<i>A. tumefaciens</i>	Tumours on plants	Stomp et al. (1990)
<i>Pinus ponderosa</i>	Ponderosa pine	<i>A. tumefaciens</i> <i>A. rhizogenes</i>	Tumours on seedlings	Morris et al. (1989), Stomp et al. (1990)
<i>Pinus taeda</i>	Loblolly pine	<i>A. tumefaciens</i>	Tumours on seedlings	Sederoff et al. (1986)
		Electroporation	Transient expression of Lux	Stomp et al. (1990), Gupta et al. (1988)
		Microprojection	Transient expression of GUS in cotyledons	Stomp et al. (1991)
<i>Pseudotsuga menziesii</i>	Douglas-fir	<i>A. tumefaciens</i>	Tumours on seedlings and Lux expression	Ellis et al. (1989)
			Tumours on seedlings and NPT II expression in tumours	Dandekar et al. (1987)
		Electroporation	Transient expression of Lux	Gupta et al. (1988)
<i>Tsuga heterophylla</i>	Western hemlock	<i>A. tumefaciens</i> <i>A. rhizogenes</i> <i>A. tumefaciens</i>	Tumours on seedlings	Morris et al. (1989)
			Tumours on seedlings	Morris et al. (1989), Morris and Morris (1990)

* All of the species listed are conifers. Abbreviations km = kanamycin, Hyg = hygromycin, GUS = β -glucuronidase, CAT = chloramphenicol acetyltransferase, Lux = luciferase, NPT II = neomycin phosphotransferase.

5.2 Problems with the genetic engineering of tree species

Although a considerable amount of knowledge has been acquired on crop plant molecular biology and genetic engineering, most of it cannot be applied directly to tree species. Trees have complex and long life cycles which render the evaluation of transgene transmission to the progeny difficult. Techniques such as accelerated flower induction could help alleviate this obstacle. Gymnosperm tree species are more difficult to work with because of their large genome (around 10^9 - 10^{10} basepairs), which makes gene manipulation and characterization tedious. In addition to these "biological" problems, genetic engineering of tree species raises special consideration at the ecological level (see section 6 for an in-depth discussion). Because they are long-live perennial and annual, potential risks to the environment associated with engineered species are higher than those associated with crop plants.

Regeneration of transgenic trees has been possible with angiosperm species (Table 12). However, the conifers appear to be recalcitrant to attempts made up to now. Several hypotheses can be put forward to explain this:

- There are fewer scientists involved in the genetic engineering of tree species than in the genetic engineering of crop species. This is the critical mass factor.
- Efficient tissue culture systems for conifers have only been developed recently with the advent of somatic embryogenesis and the improvement of organogenesis.
- There is as yet a lack of understanding of the gene structure and function of conifer species. So far, just 10 conifer gene sequences have been characterized (Table 14). Only genes isolated for crop plant engineering have been used for conifer genetic transformation. Problems encountered could be related to specific regulatory sequences (in conifer species) or to different levels of gene methylation.
- A good gene delivery system has not yet been found for conifers. *Agrobacterium* has been shown to do it (Table 13) but efficiencies are relatively low. New methods, such as microprojection, are being tested, but as yet without success.

We are confident though that, through the participation of more research groups and with

the arrival of new technologies and knowledge, transgenic conifers will be regenerated in the near future.

5.3 Potential traits for tree species

From the discussion in section 4, it is easy to identify traits with potential applications in tree species. With the current state of technology, traits will most likely be transferred to angiosperm trees in the near future.

Those angiosperms that are being used for energy farming or biomass production would benefit from traits such as *resistance to herbicide and disease* (insect, fungal, viral, and bacterial). Because such species are used in short rotation, problems associated with the development of resistant pests are less important. For fruit trees, traits associated with *fruit quality and flower sterility* could be very useful. *Flower colour modification* is a characteristic that could be exploited in ornamentals.

Generally, the introduction of pest resistance in both angiosperm and gymnosperm species that have a long life cycle is not desirable if the introduced genes are expressed constitutively. Further work will have to be carried out to ensure that the genes will be expressed only when there is a serious outbreak of the pest. Even then, there are questions about the appearance of resistant pests following long-term exposure to resistant trees. The use of *multigenic traits* could reduce this possibility.

Flower sterility has tremendous potential for long lived species. This trait will probably be required for plantations to avoid genetic pollution of, or by wild species. Strategies will have to be developed to ensure that not only is the pollen sterile (as described in section 4.11) but, as well, the female organs. In addition, sterility in trees would increase the annual yield by a significant percentage.

Growth regulator genes could be used to modify *wood quality*. Much work has to be done to examine the effect of hormone balance modifications. Another aspect that can be approached with hormone genes is *modification of apical dominance*. This could be used with ornamental tree species to modify their shape.

With increased activity in tree molecular biology, it is clear that the most interesting applications will arise not from transfer of

Table 14. Conifer DNA sequences characterized

Species	Sequence	Length and type	Reference
<i>Pinus sylvestris</i>	Chlorophyll a/b binding protein	2672 bp, cDNA nucleus	Jansson and Gustafsson (1990)
<i>Pseudotsuga menziesii</i>	Large subunit of ribulose 1,5-bisphosphate carboxylase	1855 bp, gDNA chloroplast	Hipkins et al. (1990)
	Repetitive Sequences	1266 bp, gDNA chloroplast	Tsai and Strauss (1989)
<i>Pinus concorta var. latifolia</i>	Actin	746 bp, gDNA nucleus	Kenny et al. (1988)
<i>Larix laricina</i>	Small subunit of ribulose 1,5-bisphosphate carboxylase	3667 bp, gDNA nucleus 2308 bp, cDNA nucleus	Hutchison et al. (1990)
<i>Pinus banksiana</i>	Phenylalanine ammonia lyase	1400 bp, cDNA nucleus	Campbell et al. (1991)
<i>Pinus radiata</i>	5S rRNA	524 bp, gDNA nucleus	Cullis et al. (1988)
<i>Pinus concorta</i>	<i>psbA</i>	213 bp, gDNA chloroplast	Lindholm et al. (1988)
<i>Pinus thunbergii</i>	Small subunit of ribulose 1,5- biophosphate carboxylase	915 bp, cDNA nucleus	Yamamoto et al. (1988a)
	chlorophyll a/b binding protein	978 bp, cDNA, nucleus	Yamamoto et al. (1988b)
	Large subunit of ribulase 1,5- biophosphate carboxylase	1425 bp, gDNA chloroplast	Mukai et al. (1991)
	Total	17 969 bp	

Note: g = genomic DNA, c = complementary DNA, bp = basepairs.

technology developed for agricultural crops but from original research dealing with tree species. Several examples can be cited to illustrate this point. DNA sequences for the enzymes phenylalanine ammonia-lyase and cinnamyl alcohol dehydrogenase have been characterized from conifers, opening the way to lignin modification in conifers. There is an increasing amount of research being conducted on lignin and cellulose biosynthesis in trees. Genes have been isolated (at the genomic or cDNA levels), opening the way for genetic manipulation of the pathways involved. This will be important for altering wood growth and quality. Other aspects, such as *drought*, *cold*, and *salt tolerance*, could also be tackled.

Another potentially important area of research associated with gymnosperm species is the study of the genes controlling morphogenesis. This could be critical for understanding the difficulties encountered in the tissue culture of differentiated mature tissues of conifers. One can envision the future manipulation of genes to *induce morphogenetic capacity* into tissues otherwise recalcitrant to tissue culture.

Readers should also note the advent of powerful new technologies for recombinant DNA work, such as PCR (polymerase chain reaction) and YAC (yeast artificial chromosomes), and the development of sophisticated RFLP (restriction fragment length polymorphism) methods such as RAPD (random amplified polymorphic DNA), which doubtless will have applications for both angiosperm and gymnosperm tree species. Hopefully, tree breeders will use these sophisticated techniques to obtain better breeding information and improved trees.

6. POSSIBLE ECOLOGICAL IMPLICATIONS OF GENETICALLY ENGINEERED TREE SPECIES

The potential impacts associated with the introduction of genetically engineered tree species are quite different from those associated with crop plants. Areas of concern include the development of resistant pests, the disturbance of ecological chains, the spread of monocultures through use of clonal material, the development of new weeds, and the genetic pollution of natural species. The first three concerns are associated

with the long life cycle of tree species and are, therefore, more acute when compared with those problems that could be observed with crop plants. For in-depth reviews on this subject, readers should consult Raffa (1989), Keeler (1989), and Roberds et al. (1990). Only major concepts will be highlighted here.

The most important ecological impact pertains to the introduction of genetically engineered trees with resistance to a given pest. The tremendous selection pressure due to constitutive expression of a single resistance gene would undoubtedly result in the development of a resistant pest. The possibility of this occurring could be minimized through the use of inducible gene expression, multiple-resistance genes with different mechanisms, and nonresistant trees mixed with resistant trees (genetic mosaics). However, these precautions would not eliminate the risks. The "secondary" effects of introducing a single pest resistance gene could be worse than the appearance of a resistant target pest. The effects on the ecological chains, the development of resistance of the target pest to other mechanisms of control, and the appearance of new nontarget pests are all potential consequences.

Ecological chains can also be disturbed indirectly through the effect of introducing new growth characteristics in transgenic trees. This could cause the appearance of new pests or the development of resistant pests due to the new physiology or morphology of transgenic trees. Such effects should not be underestimated and must be evaluated with regard to the environment where the transgenic trees will be located.

A third potential problem could be envisioned where transgenic trees would become undesirable weeds. It is conceivable that the introduction of a new gene, e.g., for herbicide or insect resistance, would provide transgenic trees with an ecological advantage that may allow these trees to invade ecosystems.

A fourth problem with introduction of transgenic trees is genetic pollution of natural species; that is, the newly introduced gene would be transferred by sexual reproduction from engineered trees to wild trees. This could cause serious problems in natural ecosystems where dynamic equilibria would be disturbed.

The introduction of genetically engineered trees calls for the use of clonal populations for

reforestation. In addition to the effects described above, there is the potential problem of gene pool reduction that could have serious effects. Obviously, this would diminish the adaptability of engineered species. Strategies to counteract the negative effects of a clonal population include the use of clonal mixtures and the establishment of ecological reserves. This problem is not limited to the introduction of transgenic trees, but to the use of tissue culture-derived propagules on a large scale as well.

This is a short introduction to the possible ecological implications of genetically engineered tree species. With the development of efficient methods of producing transgenic trees, the impacts will have to be studied seriously and the risks evaluated in view of the environments affected. Proper regulations will have to be developed that will take into account the differences between forest trees and crop plants.

7. REGULATIONS FOR TRANSGENIC TREE TESTING AND RELEASE IN CANADA

At the time of writing there were no regulations specifically addressing the testing and release of transgenic trees in Canada. Guidelines have been established by the Plant Seed Division of Agriculture Canada for crop plants and permits can be obtained from them. This does not, however, include tree and horticultural species. The guidelines for crop plants are not enforced by law except through the process of variety registration. However, the Canadian government intends to have proper regulations together by 1992 (as stated in Canada's Green Plan).

For transgenic plants covered by Agriculture Canada guidelines, the definition is as follows: "Genetically-modified organisms are those genetically-altered organisms that possess novel traits or characteristics which require a separate regulatory assessment of risk to human safety or the environment (e.g., pest tolerance, stress tolerance or composition changes outside the norm for the species (variety) which would present a potential risk)" (Kalous and Duke 1989). The scenario that is being proposed to regulate/guide research on and testing and

release of transgenic plants is summarized in Table 15.

Table 15. Stages of development of and possible mechanisms of controlling genetically altered plant material

Stage of development	Possible control mechanism
Laboratory and greenhouse	Guidelines
Small-scale field tests	Guidelines/advisory committee
Large-scale field tests	Regulations
Commercialization	Regulations

Source: Kalous and Duke (1989).

In the case of field testing a crop plant, the process is:

1. Submission of a letter of intention and the requested data (Table 16) to the Director, Seed Division (Food Production and Inspection Branch, Agriculture Canada, Ottawa).
2. Review by the authorities of the application on a case-by-case basis involving other agencies as needed (Health and Welfare Canada, Environment Canada).
3. Authorization by the Director, Seed Division.
4. Site inspections during and after the trial.

Because of the biological differences between crop plants and tree species, there is an urgent need to develop specific rules for testing and releasing transgenic trees in Canada. Work in this area is being carried out at Forestry Canada and guidelines or regulations are being formulated. The prerequisite for this, however, is the establishment of a seed certification act for tree seeds.

8. RESEARCH ACTIVITIES IN CANADA

There are seven groups active in genetic engineering of tree species in Canada (Table 17). Their interests are spread over both angiosperm and gymnosperm tree species and are principally aimed at developing methods of genetic transformation rather than trying to introduce valuable characteristics. At the time of writing, no transgenic trees were being tested or considered for testing in Canada (Table 18). However, in the United States, transgenic walnut and poplar are currently being tested or will be tested in the near future (Table 18).

Table 16. Information requested in applications for importing and field testing genetically transformed plant material

1.	Name of plant variety, including ability to cross-pollinate with members of the same species and native relatives, and description of plant variety
2.	Gene donor description
3.	Name of manipulated gene, identification of protein product, and gene's effect on the plant; complete gene description, including base sequence and the metabolic pathway affected
4.	Description of transformation system: i) identity of vectors and plasmid constructs ii) identification of plasmid characteristics; disarming and markers iii) description of transfer and integration mechanisms
5.	Description and results of chemical, biochemical, and statistical tests and analyses performed on the plant material
6.	Description of proposed test site i) exact map location(s) ii) size and number of plot(s) iii) quantity of seed (grams) to be planted iv) proposed control variety(s) v) isolation distances
7.	Identification of site-monitoring techniques, both during test and postharvest
8.	To what developmental stage the transgenic plants will be grown, e.g., at what stage plant material is harvested (preflowering, prematurity, etc.)
9.	Postharvest land treatment i) tillage, burning, or chemicals ii) agreement to plant no similar species on-site for a specific period (3-5 years) iii) postharvest monitoring system iv) treatment of any remaining plant material v) soil analyses, if necessary vi) volunteer plants
10.	Treatment of harvested transgenic plant material and seed
11.	Harvested seed and/or plant material i) destination/fate ii) analyses to be performed iii) determination of safety for animals and humans iv) efficacy
12.	Identification of possible environmental impact due to the field test

Source: Kalous and Duke (1989).

Table 17. Groups actively involved in tree genetic engineering in Canada

Leader(s)	Location	Species
John Carlson	University of British Columbia	<i>Populus</i> <i>Pinus</i>
Pierre Charest	Petawawa National Forestry Institute	<i>Populus</i> <i>Larix</i> <i>Picea</i>
David Dunstan	Plant Biotechnology Institute	<i>Picea</i> <i>Pinus</i>
Larry Fowke	University of Saskatchewan	<i>Picea</i>
Maurice Lalonde	Université Laval	<i>Betula</i>
Maurice Moloney/ Trevor Thorpe	University of Calgary	<i>Picea</i>
Aladar Szalay	University of Alberta	<i>Pinus</i> <i>Populus</i>

Table 18. Estimate of transgenic field trials in Canada and the United States*

Crop	Transgenic trials		Applications approved	
	(1990) Canada	(1990) USA	(1988-1990) Canada	(1987-1990) USA
Canola	8	-	26	-
Flax	2	-	10	-
Soybean	-	1	-	8
Alfalfa	1	1	4	4
Tomato	-	6	2	28
Potato	2	4	3	6
Tobacco	2	5	3	18
Cotton	0	7	-	13
Cucumber	-	1	-	2
Cantaloupe/squash	-	2	-	7
Rice	-	2	-	3
Corn	-	2	1	4
Poplar	-	-	-	1
Walnut	-	1	-	1

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